IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
HEATH, *et al.*
Serial No.: 08/699,716
Filed: 27 August 1996
For: RECOMBINANT F1-V PLAGUE VACCINE

Art Unit: 1645
Examiner: Duffy, Patricia Ann
Atty. Dckt: 003/029/SAP

AFFIDAVIT OF DAVID G. HEATH

1. I, David G. Heath, an inventor of the above-referenced application and resident of Frederick, MD, declare the following:
2. My curriculum vitae is attached.
3. Arthur M. Friedlander, George W. Anderson, Jr., Susan L. Welkos and I are joint inventors of the subject matter disclosed in the above-referenced application.
4. From **[redacted date which is before 13 March 1996]** to the end of December 1995, I conducted research and development on a plague vaccine comprising a F1-V fusion protein as a Principal Investigator as part of the Army Plague Vaccine Group.
5. During a Army Plague Vaccine Group weekly meeting sometime before **[redacted date which is before 13 March 1996]**, I volunteered to make a fusion protein comprising the F1 and V antigens (F1-V fusion) of *Yersinia pestis* for use as a new plague vaccine. On or before **[redacted date which is before 13 March 1996]**, I began working on developing an F1-V fusion protein which is evidenced on page 44 of my notebook #3487 which shows the primers and PCR plan to create the fusion protein. See Exhibit DH1.
6. On **[redacted date which is before 13 March 1996]**, I conducted the PCR experiments to fuse F1 antigen to part of V antigen (F1-V partial) which is evidenced by page 45 of my notebook #3487. See Exhibit DH2. Although it is difficult to see on this exhibit, both the BamHI/EcoRI F1 PCR fragment and the V 168-275 (321 base pairs) PCR product are present and ready for ligation or fusing. The original gel clearly shows the 321 base pair V segment in four continuous lanes. This notebook page is corroborated by the Invention Disclosure prepared and submitted by Friedlander. See Exhibit DH3 (AF5).
7. On or about **[redacted date which is before 13 March 1996]**, I sketched the plan for the F1-V partial in my notebook #3487 on page 46. This is corroborated by the Invention Disclosure prepared and submitted by Friedlander. See Exhibit DH4.
8. Before **[redacted date which is before 13 March 1996]**, I made the F1-V partial which was discussed during the Army Plague Vaccine Group meeting of **[redacted date which is before 13 March 1996]**. See Welkos' Army Plague Vaccine Group meeting notes, Exhibit DH5 (SW1).
9. On **[redacted date which is before 13 March 1996]**, I showed the construction of the PCR ligation of part of V antigen to F1 antigen and I disclosed the construction during the Army Plague Vaccine Group meeting the same day. See Exhibits DH6A and DH6B (SW5).

10. On **[redacted date which is before 13 March 1996]**, I ran the DNA gels which indicate that F1 and V partial are fused. The gels are provided in my notebook #3487 on page 56. See Exhibits DH7A and DH7B. In exhibit DH7A, it can be clearly seen on enzymatic digestion of pF1V3 a-e with EcoRI and SalI that the small 321 base pair V 168-275 segment appears along with the two other bands with the intermediate band consisting of pBluescript (the vector) and the largest band consisting of the entire F1 operon.

11. During the Army Plague Vaccine Group meeting of **[redacted date which is before 13 March 1996]**, we discussed the need for a good monoclonal antibody to test whether my clones produce F1-V partial. This is evidenced by Welkos' Army Plague Vaccine meeting notes. See Exhibit DH8 (SW2). In this immunization, F1V partial is absorbed to alhydorgel which serves as an adjuvant to stimulate the immune response in the presence of the F1-V partial protein.

12. After obtaining suitable monoclonal antibody, on **[redacted date which is before 13 March 1996]**, I ran Western blots to prove that both antibody specific for F1 antigen and antibody specific for V antigen bound independently to the F1-V fusion. See Exhibit DH9.

13. From about **[redacted date which is before 13 March 1996]** to about **[redacted date which is before 13 March 1996]**, I worked on obtaining a highly pure F1-V partial.

14. On **[redacted date which is before 13 March 1996]**, I obtained highly pure F1-V partial as evidenced by a Western blot. See Exhibit DH10.

15. On **[redacted date which is before 13 March 1996]**, I first added the F1-V partial to alhydrogel and then gave the alhydrogel F1-V partial preparations to George W. Anderson, Jr. for immunizing mice. See Exhibit DH11.

16. On **[redacted date which is before 13 March 1996]**, I planned how I would fuse F1 antigen with all of V antigen to express a fusion protein having F1 antigen fused to all of V antigen (F1-V whole). See Exhibit DH12.

17. From **[redacted date which is before 13 March 1996]**, I constructed the DNA constructs which would express F1-V whole. See Exhibit DH13.

18. On **[redacted date which is before 13 March 1996]**, I obtained highly pure F1-V whole which is evidenced by an SDS-PAGE gel of FPLC fractions of purified F1-V whole. See Exhibit DH14.

19. On **[redacted date which is before 13 March 1996]**, I ran a gel of F1, V and F1-V whole (F1VE on gel) which evidences that the F1 antigen is fused to the V antigen since F1-V is larger than V alone. See Exhibit DH15.

20. On **[redacted date which is before 13 March 1996]**, George W. Anderson, Jr. gave me the protocol for formulating the F1-V whole vaccine preparations for mouse challenge assays. See Exhibit DH16.

21. On **[redacted date which is before 13 March 1996]**, I obtained the sequencing results which evidence that the clones used to express the F1-V whole do indeed contain the DNA for the F1 antigen recombinantly fused to the DNA for all of the V antigen. See Exhibit DH17.

22. Sometime before **[redacted date which is before 13 March 1996]**, I gave the F1-V whole vaccine preparations requested by George W. Anderson, Jr. to him.

23. On **[redacted date which is before 13 March 1996]**, I prepared various alhydrogel adsorptions for George W. Anderson, Jr. for immunizing mice. See Exhibit DH18.

24. Sometime before 15 January 1996, I prepared an abstract summarizing the experiments and data on the F1-V fusion proteins which was sent to an independent review panel on 15 January 1996. A copy of the abstract is found in the AIBS Peer Review to USAMRMC Medical Biological Defense Research Program on Plague signed by Kathleen McDonough on 12 March 1996. See Exhibit DH19 (Abstract 17).

25. Abstract 17 evidences that the F1-V partial and F1-V whole had been isolated, purified and showed efficacy as a vaccine by at least 15 February 1996.

26. I left the Army Plague Vaccine Group at the end of December 1995 for an overseas assignment.

27. I have reviewed and analyzed the Titball patent and the three priority documents, UK 9505059, UK 9518946, and UK 9524825, and PCT/GB96/00571.

28. It is my opinion that prior to 13 March 1996, the filing date of PCT/GB96/00571, the inventors of the Titball patent had not conceived and/or reduced to practice a plague vaccine comprising purified F1 antigen fused to all or part of V antigen as nowhere do UK 9505059, UK 9518946, and UK 9524825 disclose isolating or purifying a protein comprising F1 antigen fused to all or part of V antigen from the host cell and other cellular components and/or administering a purified protein comprising F1 antigen fused to all or part of V antigen to a subject.

    a. In fact, UK 9518946 is the first disclosure indicating a genetic vaccine or how a host organism may be transfected with DNA for F1 antigen and V antigen to result in a live vaccine, i.e. an attenuated host organism (such as Salmonella) which produces the antigen when administered to a subject.

    b. As described in UK 9518946, the genetic vaccine or the live vaccine is administered to a subject such that the protein/antigen of interest is then produced in the subject.

    c. UK 9518946 does not describe isolating the protein/antigen of interest from the host organism and purifying the protein/antigen of interest from other cellular components prior to administration to a subject.

    d. The genetic vaccine or live vaccine described in UK 9518946 is not a purified protein comprising F1 antigen fused to all or part of V antigen which is isolated and purified from cells and other cellular components as claimed in the above-referenced application.

29. I have reviewed and analyzed the experiments and data of the Army Plague Vaccine Group and it is my opinion that the Army Plague Vaccine Group:

    a. Conceived of a fusion protein comprising F1 antigen fused to part of V by at least **[redacted date which is before 13 March 1996]**.

    b. Conceived of a fusion protein comprising F1 antigen fused to all of V by at least **[redacted date which is before 13 March 1996]**.

    c. Conceived of and reduced to practice a purified fusion protein comprising F1

antigen fused to part of V by at least **[redacted date which is before 13 March 1996]**.

d. Conceived of and reduced to practice a purified fusion protein comprising F1 antigen fused to all of V by at least **[redacted date which is before 13 March 1996]**.

e. Conceived of and reduced to practice a vaccine against plague comprising a purified fusion protein comprising F1 antigen fused to part of V by at least **[redacted date which is before 13 March 1996]**.

f. Conceived of and reduced to practice a vaccine against plague comprising a purified fusion protein comprising F1 antigen fused to all of V by at least **[redacted date which is before 13 March 1996]**.

30. I declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

David G. Heath Date: 14 Mar 2007

David G. Heath

CURRICULUM VITAE

NAME: David George Heath

DOB: February 13, 1953

RANK / A.O.C. Lieutenant Colonel / 71A
TITLE / BRANCH: Microbiologist / Medical Service

EDUCATION:

B.S. Microbiology, 1979, Indiana University, Bloomington, IN

M.S. Medical Microbiology, 1983, University of Minnesota, Minneapolis, MN

Ph.D. Microbiology, 1988, University of Minnesota, Minneapolis, MN

EMPLOYMENT:

Teaching Assistant, University of Minnesota, 1982-85

Post-Doctoral Associate
Department of Microbiology
Medical College of Ohio
Sept. 1988 - Aug. 1989

Research Fellow
Dept. of Biologic amd Materials Science
School of Dentistry
University of Michigan
Sept. 1989 - June 1992

MILITARY ASSIGNMENTS:

DATES	**POSITION/ORGANIZATION/LOCATION**
1978-1981	Environmental Health Specialist, 10th Med. Lab., Landstuhl, GER
1992-1995	Principal Investigator, Bacteriology Division, Ft. Detrick, MD
1996-1999	Chief, Clinical Microbiology, DPALS, LRMC, Landstuhl, GER
1999-2001	ABMM Post-Doctoral Fellow, U. of North Carolina, Chapel Hill, NC
2001-2004	Chief, Infectious Diseases Laboratory, DPALS, LRMC, Landstuhl, GER
2004-Present	Chief, Bacteriology, USAMRIID

AREAS OF

INTEREST:
Mechanisms of Microbial Pathogenicity
Immunology
Genetics and Molecular Biology of Bacteria and Parasites
Clinical Microbiology

PROFESSIONAL SOCIETIES:
American Society for Microbiology
Society of Armed Forces Medical Laboratory Scientists

FELLOWSHIPS:
USPHS Predoctoral Fellow in Microbiology\Cancer Research

Post-Doctoral Fellowship, American Heart Association,
Sept. 19888- Aug. 1989

MILITARY EDUCATION:
91S Environmental Health Specialist School (1978)
Officer Basic Course (1992)
Medical Defense of Biological Warfare (1993)
Medical Management of Chemical Casualties (1993)
Officer Advanced Course (1996)
Combined Arms and Services Staff School (1996)
Command and General Staff College (1999)

AWARDS:
Meritorious Service Medal (1995)
Meritorious Service Medal (1999)
Meritorious Service Medal (2004)

SPECIALIZATION/TECHNIQUES:

Experience in gene cloning, protein purification, recombinant DNA manipulation and DNA sequencing. Additional experience includes transposon mutagenesis, manipulation of Gram positive cloning vectors, polymerase chain reaction with primer synthesis and various immunoassays. Animal immunizations, serum characterization for antibody expression, Western blotting. Experience in recombinant vaccine development against the pneumonic form of *Yersinia pestis* leading to a patent application.

PUBLICATIONS:

JOURNAL ARTICLES (FIRST AUTHOR):

Heath, David.G., Kathy Hohnecker, Charlene Carriker, Kelly Smith, Jonathon Routh, John J. LiPuma, Robert M. Aris, David Weber, and Peter H. Gilligan. 2002. Six-year molecular analysis of *Burkholderia cepacia* complex isolates among cystic fibrosis patients at a referral center for lung transplantation. **J. Clin. Microbiol.** 40: 1188-1193.

Heath, David G., George W. Anderson Jr., J. Matthew Mauro, Susan L. Welkos, Gerard P. Andrews, Jeffery Adamovicz, and Arthur M. Friedlander. 1998. Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. **Vaccine.** 16: 1131-1137.

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Heath, David G. and P. Patrick Cleary. 1987. Cloning and expression of the gene for an Fc-receptor protein from a group A streptococus. **Infect. Immun.** 55: 1233-1238.

JOURNAL ARTICLES (OTHER THAN FIRST AUTHOR):

Powell BS, Andrews GP, Enama JT, Jendrek S, Bolt C, Worsham P, Pullen JK, Ribot W, Hines H, Smith L, Heath DG, Adamovicz JJ. 2005. Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. **Biotechnol Prog.** 21(5):1490-510.

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Christopher, George W., D.G. Heath and K.L. Glass. 2002. A severe case of NLV gastroenteritis: Case report and lessons learned. Amedd J. Oct/Nov.

Brecher, M.E., D. Heath, S.N. Hay, S. Rothenberg L.C. Stutzman. 2002. Evaluation of a new generation culture bottle with the bact/alert 3D microbial detection system with 9 common contaminating organisms in platelett components. ***Transfusion*** 42: 774-779.

Aris, R.M., J.C. Routh, J.J. LiPuma, D.G. Heath and P.H. Gilligan. 2001. Lung transplantation for cystic fibrosis patients with *Burkholderia cepacia* complex. Survival linked to genomovar type. ***Am. J. Respir. Crit. Care Med.*** 164: 2102-2106.

Brecher, M.E., N. Means, C.S. Jere, D. Heath, S. Rothenberg, and L.C. Stutzman. 2001. Evaluation of an automated culture system for detecting bacterial contamination of platelets: an analysis with 15 contaminating organisms. ***Transfusion*** 41: 477-482.

Benner, G.E., G.P. Andrews, W.R. Byrne, S.D. Strachan, A.K. Sample, D.G. Heath and A. M. Friedlander. 1999. Immune response to *Yersinia* outer proteins and other *Yersinia pestis* antigens after experimental plague infection in mice. ***Infect. Immun.*** 67: 1922-1928.

Brandler, P., K.U. Saikh, D. Heath, A. Friedlander and R. G. Ulrich. 1998. Weak anamnestic responses of inbred mice to *Yersinia* F1 genetic vaccine are overcome by boosting with F1 polypeptide while outbred mice remain nonresponsive. ***J. Immunol.*** 161: 4195-4200.

Krakauer, Teresa and David Heath. 1998. Lack of IL-1 receptor antagonistic activity of the capsular F1 antigen of *Yersinia pestis*. ***Immunol. lttrs.*** 60: 137-142.

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Andrews, Gerard P., David G. Heath, George W. Anderson Jr., Susan L. Welkos, and Arthur M. Friedlander. 1996. Fraction 1 capsular antigen (F1) purification from *Yersinia pestis* CO92 and from an *Escherichia coli*

recombinant strain and efficacy against lethal plague challenge. ***Infect. Immun.*** 64: 2180-2187.

Friedlander, Arthur M., Susan L. Welkos, Pat L. Worsham, Gerard P. Andrews, David G. Heath, George W. Anderson Jr., M.L. Pitt, John Estep, and Kelly Davis. 1995. Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*. ***Clin. Infect. Dis.*** 21: Suppl 2: S178-181.

Vickerman, Megan M., David G. Heath, and Don B. Clewell. 1993. Construction of recombination-deficient strains of *Streptococcus gordonii* by disruption of the *recA* gene. ***J. Bacteriol.*** 175: 6354-6357.

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Boyle, M.D.P., E.L. Faulmann, R.A. Otten, and D.G. Heath. 1990. Streptococcal immunoglobulin binding proteins. In Elia Ayoub and Gail H. Cassel (eds.), Microbial Determinants of Virulence and Host Response. American Society for Microbiology Publications, Washington, D.C.

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Ph. D DISSERTATION

David G. Heath, Ph.D. Dissertation, "Characterization of an Fc-receptor gene from an M-type 76 strain of group A streptococci." University of Minnesota, Minneapolis, MN 1988.

PRESENTATIONS

Heath, David G. and P. P Cleary. Cloning and expression of an Fc-binding protein from group A streptococci M-type 76 in Escherichia coli. *Second ASM Conference on Streptococcal Genetics*. Miami Beach, FL. May 1986

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Heath, D.G., F.An, S. Flannagan, K. Tanimoto, and D.B. Clewell. Phase variation in the conjugation functions of *Enterococcus faecalis* plasmid pAD1 is due to amplification of direct repeats. *93rd General Meeting of the American Society for Microbiology.* May, 1993. Atlanta, GA.

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Andrews, G., G. Howe, D. Heath, C. See, A. Maurelli, and A. Friedlander. Temperature-dependent expression of *Yersinia pestis* fraction 1 capsular antigen (F1) is controlled by a homolog of the thermoregulatory loci, *hns* of *Escherichia coli* and *virR* of *Shigella flexneri. 94th General Meeting of the American Society for Microbiology.* May, 1994. Las vegas, Nevada.

Heath, D.G., G.P. Andrews, G.B. Howe, S. Chatmon, and A. Friedlander. Purification and characterization of the fraction 1 capsular antigen (F1) from *Yersinia pestis* CO92 and an *Escherichia coli* F1 recombinant. *94th General Meeting of the American Society for Microbiology.* May, 1994. Las Vegas, Nevada.

An, F.Y., D.G. Heath, and D.B. Clewell. pAD1 phase variation in *Enterococcus faecalis*: Amplification of direct repeats increases transfer frequency. *4th International ASM Conference on Streptococcal Genetics.* May, 1994. Santa Fe, NM

Fritz, D., K. Davis, D. Heath, S. Welkos, L. Pitt, and A. Friedlander. Detection of *Yersinia pestis* capsular protein by light and electron microscopy using monospecific polyclonal rabbit anti-F1 antibody. *Annual Meeting of the American College of Veterinary Pathologists.* Nov., 1994. Montreal, Quebec, Canada.

Anderson, G.W. Jr, C. Yan, M. Kende, S.L. Welkos, D.G. Heth and A.M. Friedlander. Efficacy elicited by the fraction 1 (F1) antigen encapsulated in poly(lactide-co-glycolide) microspheres against *Yersinia pestis. 96th General*

Meeting of the American Society for Microbiology. May 1996. New Orleans, LA

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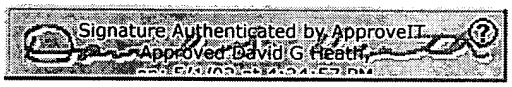
Heath, D.G., K. Hohneker, C. Carriker, K. Smith, J. Routh, J.J. LiPuma, R. M. Aris, D. Weber and P.H. Gilligan. Molecular epidemiology of *Burkholderia cepacia*: The UNC Experience. *15th Annual North American Cystic Fibrosis Conference.* Oct. 2001. Orlando, FL

Aldous, W. and D.G. Heath. Molecular Diagnosis of Infectious Diseases. Oral Presentation. *26th Annual Meeting of the Society of Armed Forces Medical Laboratory Scientists.* Mar. 2002. Spokane, WA.

PERSONAL REFERENCES PROVIDED ON REQUEST



David G. Heath, Ph.D.
LTC MS
Chief, Bacteriology Division
USAMRIID

Exhibit DH1

REDACTED

Pick single colony isolated of Antigua CCR-6VH-pBlue B/E and of Barbados CCR-6VH pBlue B/E (2 of each)

- grew up 15 ml cultures ON - will have Sam make Qiagin plasmid DNA + sequence using universal + reverse primer or perhaps just universal primer

REDACTED

Rabbits were bled by VMD personnel. Took serum and stored @ 4°C.

Designed primers to segment part of V to F1 capsule had Kristin make 4 primers:

BAMHIF1 5' 5' GAA AAA GAA TCA CAG GAT CGT TTC 3'

EcoRI P1 Rev 5' CTC GAA TTC TTG GTT AGH TAC GGT 3'

V168 For 5' CAC GAA TTC TCA GTT ATT CAA GCC G 3'

V275 REV 5' GTG GGT CGA CTC AAT CCG AGC AGG TGG T 3'

Basic plan is to PCR pYPR1 to get F1 operon from BAMHI site to very end of F1 capsule gene

B——E

for V have from 168 to 275 amino acids

Eco
168 Sal I = 5 b/p codon

ligate the 2 PCR products after B/E + E/Sal digestion to get a B/Sal fragment. Then ligate into B/Sal of pBluescript

B Sal

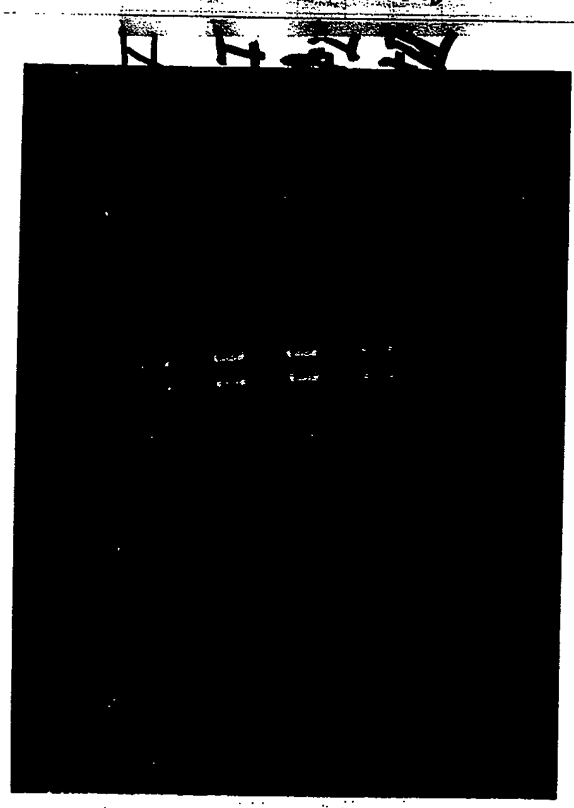
- then ligate new B/Sal piece to E/B small fragment of pYPR1

Fl-V

B ——— / Sal + E —— // —— B = E ———— / B Sal

so now ligate into E ——> Sal digested pBluescript & grow at 30 or 37°C

REDACTED Had some do Qiagen Plasmid isolation of 4 recombinants c LCK-GVH operon. He then digested c E/B & ran on agarose. He also sequenced insert using reverse & forward primers



Results: The four isolates look like they have about the right sized insert ~2.1KB.

REDACTED set up Ouchterlony using rabbit sera vs. F1

rabb. #1 ○ ○ Anti plaque rabbit

rabbit #2 ○ ○ F1 40% cut

using 4 week post injection sera (also 1 boost @ 2nd week)

Rabbit # 1 = 7F7D1D3A12
Rabbit # 2 = 7F7D1D716A

Exhibit DH2

REDACTED

PCR reactions of pYPR1 x A1-A2, P1-P2 to do gene splicing

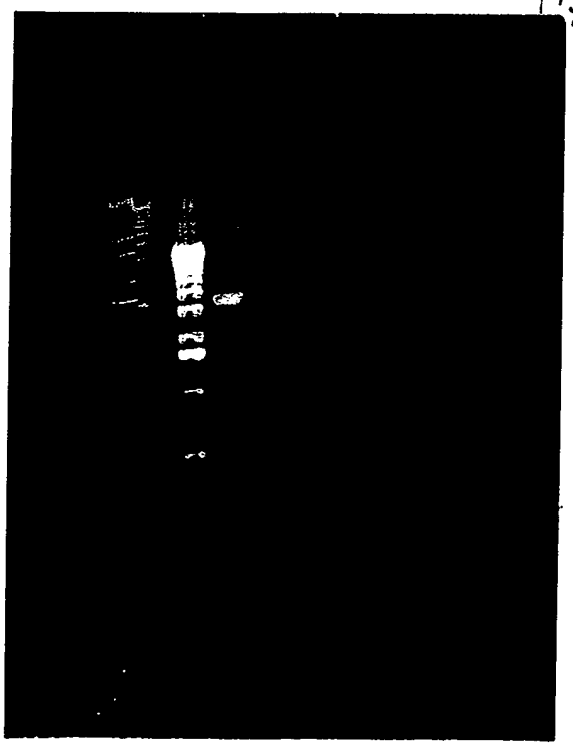
- R1 BAMHI-FLendEco
  - 1 ul template (pYPR1 712 ng/ul)
  - 10 ul 10 x Reaction Buffer
  - 2 ul dATP
  - 2 ul dTTP
  - 2 ul dCTP
  - 2 ul dGTP
  - 2 ul BAMHI For
  - 2 ul Eco Rev
  - 0.5 ul Taq Poly
  - 76.5 ul dH_2O
  - 100 ul

A1 - V168-275
- 1 ul Antigen #1 template
- 10 ul React. Buffer
- 2 ul dATP
- 2 ul dTTP
- 2 ul dCTP
- 2 ul dGTP
- 2 ul V168
- 2 ul V275
- 0.5 ul Taq
- 76.5 ul dH_2O

A2 - V168-275
Same as A1

P1 - V168-275
Same as A1

P2 - V168-275
Same as A1



Results: got a 3.5KB F1 prod. & a ~320 bp prod. for V. should get a 321 bp prod. for V and 3,644 bp prod. for F1

For total clone = E/B small the size should be
- 4.3 for small E/B of pYPR1
- 3.6 for F1 PCR prod
- 321 bp for V prod.
- 2.9KB for pBluescript

~11.1 KB total product

Exhibit DH3

DISCLOSURE RECEIVED AT LEGAL DATE: _____
FROM ORTA: 16 APR 96
LOGGED IN BY: _____
LOG NUMBER: RUD 9608

REDACTED

APPENDIX A

DEPARTMENT OF THE ARMY
UNITED STATES OF AMERICA

INVENTION DISCLOSURE

(THIS FORM AND ACCOMPANYING DRAWING AND DESCRIPTION SHEETS ARE TO BE COMPLETED FOR EACH INVENTION PROMPTLY FORWARDED TO THE PATENT ACTIVITY(8))

PATENT ACTIVITIES DOCKET NO. 16-08

ASSIGNED TO:

SHORT TITLE OF INVENTION
Recombinant F1-V Plaque Vaccine

FULL NAME(S) OF INVENTOR(S) (FIRST)	(MIDDLE INITIAL)	(LAST)	HOME ADDRESS(ES)	(DUTY) TEL. NO. AREA CODE
(1) David	G.	Heath	Bacteriology Division	(301) 619-7341
(2) Arthur	M.	Friedlander	USAMRIID, Bldg. 1425	7341
(3) George	W.	Anderson	Fort Detrick	4433
Susan	L.	Welkos	Frederick, MD 21702-5011	4430

INFORMATION AND DATES CONCERNING THIS INVENTION

NEEDED IN THE EVENT OF A CONTEST OF PRIORITY OF INVENTION IN THE U.S. PATENT AND TRADEMARK OFFICE. ALL RECORDS CITED SHOULD BE DATED AND SIGNED BY TWO INDEPENDENT WITNESSES WHO HAVE READ AND UNDERSTOOD THE MATERIAL.

(4) ON WHAT DATE DID YOU FIRST THINK OF THIS INVENTION (WHAT RECORDS SHOW THIS?): REDACTED — Laboratory Notebook

(5) GIVE DATE OF AND IDENTIFY EARLIEST SKETCH OR DRAWING: REDACTED

(6) WHEN/WHERE AND TO WHOM DID YOU MAKE THE FIRST DISCLOSURE TO OTHERS OF THE INVENTION EITHER ORALLY OR IN WRITING? See enclosure

(7) DESCRIBE DETAILS OF ANY WORK OR TESTS DONE TO PRODUCE OR OPERATE THE INVENTION GIVE DATES AND WITNESSES (USE OTHER PAGES IF NECESSARY): REDACTED

(8) DESCRIBE AND GIVE DATES OF ANY OTHER SKETCHES, DRAWINGS OR REPORTS PERTINENT TO THIS INVENTION

USE, SALE OR PUBLICATION

NEEDED TO ESTABLISH THE DATE OF ANY PRINTED PUBLICATION, PUBLIC USE OR SALE. SINCE NO PATENT APPLICATION MAY BE FILED AFTER ONE YEAR FROM SUCH DATE.

(9) IF INVENTION HAS BEEN SOLD OR USED FOR PROFIT- WHEN AND TO WHOM DISCLOSED OR WHEN AND HOW USED? Not sold or used for profit

(10) HAS A DESCRIPTION OF THIS INVENTION BEEN MADE AVAILABLE TO PERSONS OUTSIDE THE ARMY? (WRITTEN OR ORAL) IF SO, HOW AND WHEN AND WAS USE RESTRICTED? See enclosure

POTENTIAL MARKET INFORMATION

NEEDED FOR POSSIBLE MARKETING INVESTIGATIONS AND AS AN AID TO POTENTIAL LICENSING TO OTHERS.

(11) DESCRIBE ANY POTENTIAL OR EXISTING MARKET FOR SALE OR LICENSE OF THIS INVENTION

A. GOVERNMENT: Vaccine against plague

B. COMMERCIAL: Vaccine against plague

C. IDENTIFY ANY KNOWN FIRMS OR VENDORS WHO MAY BE INTERESTED IN THE INVENTION
Greer Laboratories, Lenoir, NC

CONTRACT INFORMATION

A DETERMINATION OF RIGHTS IN THIS INVENTION WILL BE NECESSARY. (SEE AR 27-60)

(12) IF THIS INVENTION WAS FIRST CONCEIVED OR CONSTRUCTED IN CONNECTION WITH:

(A) MY DUTIES AS A GOVERNMENT EMPLOYEE

B. MY WORK UNRELATED TO MY DUTIES AS A GOVERNMENT EMPLOYEE (PRIVATE, OFF DUTY ACTIVITIES)

C. MY DUTIES AS A GOVERNMENT EMPLOYEE & WORKING WITH A CONTRACTOR

D. NEITHER A, B OR C, EXPLAIN

FOREIGN FILING CONSIDERATION

NEEDED TO DETERMINE THE POTENTIAL WORLDWIDE USE FOR THE INVENTION.

(13) INDICATE THE POTENTIAL FOR USING THIS INVENTION IN FOREIGN COUNTRIES

☐ POOR

☐ GOOD

☒ EXCELLENT

SECURITY CLASSIFICATION

(13A) PLEASE INDICATE THE SECURITY CLASSIFICATION IF KNOWN

☐ CLASSIFIED LEVEL

☒ UNCLASSIFIED

☐ CLASSIFICATION UNKNOWN

DEPARTMENT OF THE ARMY
UNITED STATES OF AMERICA

INVENTION DISCLOSURE

(DRAWING AND DESCRIPTION SHEET)

PATENT ACTIVITIES DOCKET NO.

(14) PROVIDE THE FOLLOWING INFORMATION CONCERNING THE DISCLOSED INVENTION AND IN THE INDICATED SEQUENCE:

A. SPECIFICALLY DESCRIBE THE INVENTION AND ITS OPERATION. YOU MAY USE AND ATTACH COPIES OF SKETCHES, PRINTS, PHOTOGRAPHS, PAPERS AND ILLUSTRATIONS, WHICH SHOULD BE SIGNED, WITNESSED AND DATED. USE NUMBERS AND DESCRIPTIVE NAMES IN DESCRIPTIONS AND DRAWINGS.

B. STATE THE ADVANTAGES OF THE INVENTION OVER PRESENTLY KNOWN DEVICES, SYSTEMS OR PROCESSES.

C. DISCUSS THE PROBLEMS WHICH THE INVENTION IS DESIGNED TO SOLVE, REFERRING TO ANY PRIOR INVENTION OF A SIMILAR NATURE WITH WHICH YOU MAY BE FAMILIAR.

D. LIST ALL KNOWN AND OTHER POSSIBLE USES FOR THE INVENTION.

E. LIST THE FEATURES OF THE INVENTION THAT ARE BELIEVED TO BE NOVEL.

USE AS MANY OF THESE SHEETS AS NECESSARY AND ATTACH TO COMPLETED INVENTION DISCLOSURE

See attached enclosure

SIGNATURE(S) AND ORGANIZATION OF INVENTOR(S) (USE INK) DATE: THE DESCRIBED INVENTION HAS BEEN WITNESSED, READ, AND UNDERSTOOD BY: DATE:

(15) _____ (18) _____

ORGANIZATION USAMRIID

(16) _____ (19) _____

ORGANIZATION USAMRIID

(17) _____ (20) _____

ORGANIZATION USAMRIID

(18) John J Wilson
USAMRIID

* NOTE: THIS FORM AND ANY OMITTED INFORMATION BECOMING AVAILABLE AT A LATER TIME SHOULD BE FORWARDED TO:

HQDA CHIEF, INTELLECTUAL PROPETY DIV. OFFICE OF THE JUDGE ADVOCATE GENERAL DEPT. OF THE ARMY WASHINGTON, D.C. 20310 DARCOM ATTN: PATENT COUNSEL; OR CHIEF OF ENGINEERS ATTN: PATENT COUNSEL

Invention Disclosure: Recombinant F1-V plague vaccine

(6) First disclosure was in a conversation with Richard Titball, CBDE, Porton Down, England during the American Soc. of Microbiology Meeting sometime during 21-25 May, 1995 in Washington, DC

(10)

a. oral communication in confidence to Richard Titball, American Soc. of Microbiology Meeting sometime during 21-25 May, 1995 in Washington, DC

b. approximately 1/15/96, written abstract sent to Tom Schwan, Rocky Mountain Labs, Hamilton, MT; Kathleen McDonough, David Axelrod Institute, Albany, NY; Dorothy Pierson, University of Colorado, Denver, CO in confidence for review of Army Plague Research program

c. written communication 1/24/96 submitted to the journal Nature

d. oral presentation on 2/15/96 at review of Army Plague Research program, Frederick, MD

e. written communication 3/19/96 submitted to the journal Science

(14)

A recombinant *Yersinia pestis*-derived F1 capsule and V antigen fusion protein.

A. The invention is a fusion protein made up of two proteins derived from *Yersinia pestis*: the F1 capsule antigen (F1) and the V antigen. The process of constructing the fusion protein required several intermediate steps. The first step called for creating a polymerase chain reaction (PCR) product consisting of part of the F1 operon and the F1 structural gene (*caf1*) open reading frame (ORF) from which the stop codon was removed (Figure 1A). The *Bam* HI/*Eco* RI restricted, F1-containing, PCR product was then ligated into the smaller isolated *Eco* RI/*Bam* HI fragment of pYPRI to create pF1LZ (Figure 1B). Next, a small internal segment of the V antigen ORF (Figure 1C) was generated by PCR and ligated into the *Eco* RI (partial digest) and *Sal* I digested pF1LZ to create pF1V3a (Figure 1D). pF1V3a then served as the template DNA in a PCR reaction to create a PCR product containing the F1 structural gene ORF fused, in frame, with the

internal V segment. This PCR product was restricted with *Nde* I and *Bam* HI and ligated into pET19b (Novagen, Inc.) to create pF1Vs (Figure 1E). The V segment and the small *Bam* HI/*Pst* I fragment from the original plasmid vector, pET19b were removed from pF1Vs (Figure 2A) and replaced with the entire V antigen ORF (Figure 2 B) in a ligation reaction (Figure 2C) which also included the small *Bam* HI/*Pst* I fragment from pET19b to create pF1V (Figure 2D). pF1V DNA was used to transform *Escherichia coli* strain BLR (Novagen) and expression of the fusion protein was then shown to occur upon induction with isopropylthio-β-galactoside (IPTG). Expression of a protein of the appropriate size for this fusion protein (58 kDa) was demonstrated by SDS-polyacrylamide gel electrophoresis (Figure 3A). The invention has been designated F1-V.

1. To purify the F1-V fusion protein, *E. coli* strain BLR containing the plasmid pF1V was grown overnight in a small shaking flask using 5 ml of LB broth containing 100 µg/ml of carbenicillin. The overnight culture was then centrifuged at 5000 x g to pellet the cells and resuspended in fresh LB/carbenicillin. One ml of the fresh suspension was used to inoculate 1 liter of LB/carbenicillin and the culture was rotated at 225 rpm and allowed to grow at 37°C for 4 to 5 hr (OD_{600} = 1). The temperature was then lowered to 26°C and IPTG was added to 1 mM final concentration at which time the culture was allowed to rotate at 225 rpm for an additional 2 hr. The cell culture was then centrifuged and cell pellets were resuspended in 40 ml of 1 x Binding Buffer (Novagen, Inc.). The suspension was then subjected to sonication (six 30 sec bursts) and the cell debris was removed by high-speed centrifugation (39,000 x g) for 20 minutes. The supernatant was removed and subjected to ultrafiltration (0.45µ filter) after which it was divided into 10 ml aliquots for storage at - 70°C. The frozen supernatant was allowed to thaw on ice and subjected to fast protein liquid chromatography (FPLC) using a Ni^{2+} chelation resin (Novagen, Inc.). The bound fusion protein was released from the resin after exposure to an imidazole gradient and fractions containing the fusion protein were pooled and buffer exchanged, by dialysis, into 20mM Tris, pH 7.6, 0.5 mM EDTA. The pooled protein was then subjected to a further round of FPLC (to remove endotoxin) using a Mono Q (Pharmacia) ion-exchange column. The purified fusion protein was then tested for endotoxin content using the Limulus amebocyte lysate assay (Sigma). The purified F1-V purified protein was subsequently shown to bind antibody

directed against either the F1 antigen or antibody directed against V antigen (Figure 3b, 3c).

2. The value of the F1-V protein was demonstrated by its ability to protect experimental animals against infection with *Yersinia pestis*, the causative agent of plague. Most forms of naturally occurring plague are due to F1 capsule containing (F1+) strains of *Y. pestis.* However, F1- or deficient plague strains have been isolated from natural sources and from a human case, and are virulent in experimental infections of mice and non-human primates.

In two separate experiments (Table 1), mice immunized with 13.6 μg of F1-V were protected (90-100% survival) against a subcutaneous challenge with a moderate (57 LD_{50}) or high (1.1×10^6 LD_{50}) dose of an F1- *Y. pestis* strain, C12, while all control animals died. Animals given 10 μg of V (equivalent to the same amount of V as in 13.6 μg of F1-V) were afforded the same degree of protection (90% survival) against the high-dose challenge. Another group of animals immunized with 27.2 μg of F1-V completely (100%) survived the high-dose challenge. In a separate experiment (Table 3), animals given just one immunization of 60 μg of F1-V were completely protected against subcutaneous challenge with a high dose of C12, while the licensed human vaccine gave no protection.

We next determined the efficacy of F1-V against pneumonic plague induced by an aerosol challenge (Table 2). Groups of mice immunized with 13.6 or 27.2 μg of F1-V were completely protected (100% survival) against a moderate (91 LD_{50}) or high (545-636 LD_{50}) aerosol challenge dose of the F1- *Y. pestis* strain, C12. In marked contrast, the current human, whole-cell plague vaccine USP, failed to prevent fatal pneumonic plague; none of eight challenged animals survived.

We next determined the efficacy of the F1-V protein in protecting mice against infection with plague strains containing the F1 capsule. Table 3 shows that a single dose of 60 ug of F1-V completely protected mice against a subcutaneous challenge with the F1+ CO92 strain. In contrast the licensed human vaccine protected only 4 of 10 animals. Moreover, while previous data showed that 2 doses of the current human plague vaccine significantly protects mice against a subcutaneous challenge with CO92, it does not protect animals after an aerosol challenge but just delays the time to death (Pitt et al. 1994 Annual Meeting, Amer. Soc. Micro. Abstract #E45, Las Vegas, NV). Furthermore,

the same study showed that the licensed vaccine did not even delay the time to death in non-human primates exposed by aerosol to CO92. Table 2 shows that immunization with 2 doses of F1-V completely protects animals against an aerosol challenge with an F1+ *Y. pestis* strain, CO92, with 10 of 10 animals surviving. Thus the F1-V vaccine, in contrast to the current licensed vaccine, protects mice against pneumonic plague with both F1+ and F1-*Y. pestis* strains, a more difficult form of the disease to protect against.

B. The invention was designed to be used in a vaccine affording protection against plague, due to exposure to the infectious agent *Yersinia pestis.* The advantages of using this fusion protein over the present whole cell vaccine are as follows:

1. The current licensed vaccine does not protect mice against subcutaneous challenge with F1- strains of *Y. pestis*, which have been shown to cause fatal disease in both humans and experimental animals infected by a peripheral, non-respiratory route. The new F1-V vaccine does protect mice against bubonic plague caused by subcutaneous challenge with F1- organisms.
2. The current licensed vaccine does not protect mice against pneumonic plague induced by aerosol challenge with F1- strains of *Y. pestis*. The new F1-V vaccine does protect mice against pneumonic plague caused by aerosol challenge with F1- strains.
3. The current licensed vaccine does not protect mice against pneumonic plague when challenged by the respiratory route with F1+ strains of *Y. pestis*. The new F1-V vaccine does protect mice against pneumonic plague caused by aerosol challenge with F1+ strains.
4. The new F1-V vaccine is expected to protect humans against pneumonic plague produced by strains of *Y. pestis*, either naturally occurring or genetically engineered, which may be altered in their content or composition of V antigen, but which still contain F1. This is because the F1-V vaccine also contains F1. The current licensed vaccine does not protect against pneumonic plague induced by either F1- or F1+ organisms when given by the aerosol route.
5. The new F1-V vaccine is composed of two antigens, both of which have been shown to be protective. It is anticipated that the combination of both antigens may provide better protection against F1+ strains than

either F1 or V when used alone as vaccines. This is possible because the immunity induced by F1 and by V occur by different mechanisms which may be additive or synergistic.

6. Approximately 8% of humans immunized with the current licensed human plague vaccine fail to develop an immune response to F1 (Marshall et al. J. Inf. Dis. 129:S26-S29, 1974). These non-responders may well be at risk for development of plague. The inclusion of two different protective antigens in the same vaccine will help to eliminate the problem of non-responders and so increase the overall efficacy of vaccination in a human population.

7. The new F1-V vaccine is composed of highly purified recombinant proteins which are very well defined. This contrasts with the present human licensed vaccine composed of whole bacteria. The nature of the protective immunogen(s) in the present vaccine is completely unknown. The present vaccine in known to contain and induce antibodies to F1 but it does not induce antibodies to V antigen in mice, suggesting that V antigen is absent. Furthermore, it is anticipated that the highly purified new F1-V vaccine will be significantly less reactogenic in humans than the present human licensed vaccine, which may contain unnecessary bacterial components responsible for its untoward side effects.

8. The F1-V protein was constructed so that a single protein could be purified as a vaccine component rather than having to purify F1 and V antigen separately. The purification of a single protein as opposed to two separate proteins could result in considerable savings when manufacturing a vaccine.

C. This invention is designed to solve the problem of protecting humans against both bubonic and pneumonic forms of plague caused by infection by the subcutaneous and aerosol routes, respectively, with either F1+ or F1- plague organisms, or with strains which may vary in their V antigen.

The current licensed human vaccine protects mice against subcutaneous challenge with F1+ strains, but only delays the time to death of mice challenged by the aerosol route. The vaccine has no protective effect and does not delay the time to death in the non-human primate exposed to F1+ organisms.

The current licensed human vaccine has no significant effect on survival of mice challenged with the F1- C12 strain by either the subcutaneous or the aerosol route.

Thus the current licensed human vaccine would be expected to be ineffective against pneumonic plague caused by either F1+ or F1- strains, or bubonic plague produced by F1- strains of *Y. pestis.*

D. Known or possible uses of this invention include the following: 1) The fusion protein could be used as a vaccine to protect against bubonic or pneumonic plague due to both F1+ and F1- strains of *Y. pestis* or strains which may vary in their V antigen content.

E. This invention is novel because it is a single constructed protein composed of two unique proteins, the entire F1 capsule antigen and V antigen. It induces an immunological response against both the F1 protein and V antigen. It is also novel because it includes 2 protective immunogens in the same vaccine.

TABLE 1 Efficacy of F1-V vaccination against a lethal subcutaneous *Y. pestis* infection of mice

Treatment Group[a]	Strain	LD_{50}[b]	Survivors/Total
Alhydrogel alone	C12	57	0/10
13.6 µg F1-V	"	"	10/10
Alhydrogel alone	"	$1.1x10^6$	0/10
10 µg V	"	"	9/10
13.6 µg F1-V	"	"	9/10
27.2 µg F1-V	"	"	10/10

[a] For all groups, 8-10 week old female Swiss Webster (Hsd:ND4) mice (Harlan Sprague Dawley) were immunized subcutaneously on day 0 and day 28 with 0.2 ml of the indicated vaccine preparation. The F1-V and V proteins were each separately adsorbed to Alhydrogel, 1.3% (aluminum hydroxide gel adjuvant, Superfos Biosector).

[b] Mice were challenged with the $F1^-$, C12 strain, prepared as previously described (Welkos et al. Contrib. Microbiol. Immunol. 13:298-305, 1995), at day 78 after the initial antigen administration.

TABLE 2. Efficacy of F1-V vaccination against a lethal aerosol *Y. pestis* infection of mice

Treatment Group[a]	Strain	LD_{50}[b]	Survivors/Total	Geometric mean antibody titer[c] F1	V
Alhydrogel alone	C12	91	0/9	NT[d]	NT
13.6 μg F1-V	"	"	10/10	NT	NT
Alhydrogel alone	"	545-636	0/14	<640	<640
10 μg V[e]	"	545-636	8/10	NT	655,360
13.6 μg F1-V	"	545-636	10/10	66,540	432,376
27.2 μg F1-V	"	545-636	10/10	108,094	432,376
Plague USP[f]	"	545-636	0/8	55,738	<640
Alhydrogel alone	CO92	761	1/10	NT	NT
13.6 μg F1-V	CO92	761	10/10	NT	NT

[a]For all groups, 8-10 week old female Swiss Webster (Hsd:ND4) mice were immunized subcutaneously on day 0 and day 28 with 0.2 ml of the indicated vaccine preparation.

[b]Mice were challenged with inocula prepared as described in Table 1 at day 78 after the initial antigen administration. Aerosol exposures were performed in a nose-only exposure chamber with a dynamic small-particle aerosol as previously described (Welkos et al. Contrib. Microbiol. Immunol. 13:298-305, 1995). The apparatus was configured to challenge a maximum of 27 mice per exposure. Mice from several groups were divided between exposure runs to minimize differences among the treatment groups resulting in a dose challenge range.

[c]Serum obtained on day 58 after the initial immunization was assayed for anti-F1 and anti-V IgG antibody by ELISA on individual animals and group geometric mean titers determined. Titers were determined as the reciprocal of the maximum dilution giving an absorbance greater than 0.1 units after subtraction of nonspecific binding in normal serum.

[d]Not tested.

[e]Because F1-V was exposed to urea during purification, we also exposed this preparation of V to urea. V in PBS was buffer exchanged into 1x Binding Buffer

(Novagen) containing 6 M urea and placed at 4°C for 4 h after which urea was removed by dialysis as indicated for F1-V. V concentration was then determined and V was adsorbed to Alhydrogel.

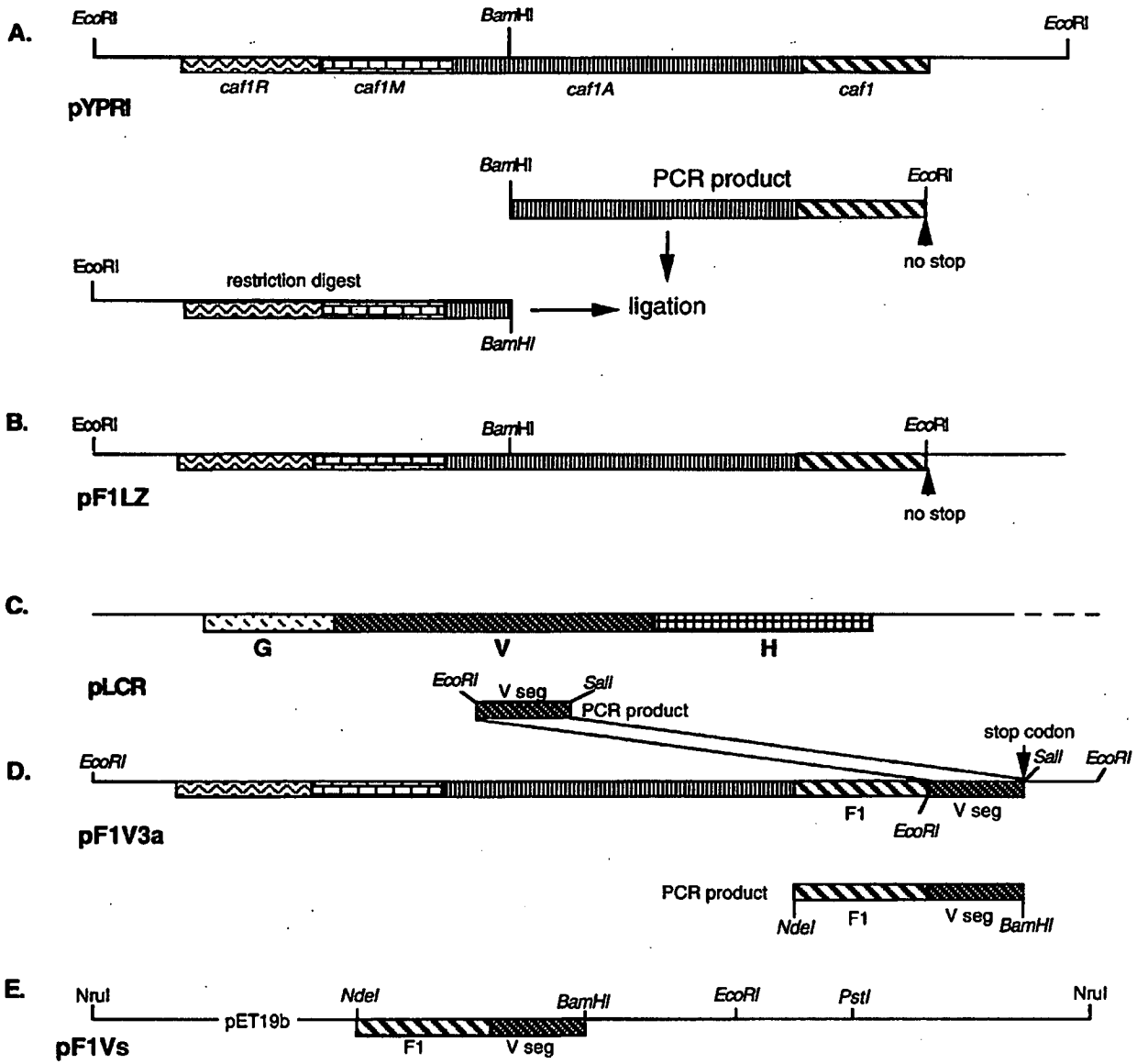
[f]The licensed, human, whole-cell plague vaccine United States Pharmacopeia (USP) was obtained from Greer Laboratories (Lenoir, NC).

TABLE 3. Survival of outbred mice after a single subcutaneous immunization followed by subcutaneous challenge.

Treatment Group[a]	Strain	LD_{50}[b]	Survivors/Total
Alhydrogel alone	CO92	5,750	0/10
Plague USP (Greer)	"	"	4/10
60.0 µg F1-V	"	"	10/10
Alhydrogel alone	C12	16,300	0/10
Plague USP (Greer)	"	"	0/10
60.0 µg F1-V	"	"	10/10

[a]For all groups, Hsd:ND4 Swiss Webster female 8-9 week old mice were immunized subcutaneously on day 0 with 0.2 ml of the vaccine preparation.
[b]Challenge was at day 44 postimmunization.

FIGURE 1.


A.
EcoRI
BamHI
EcoRI
caf1R
caf1M
caf1A
caf1
pYPRI
BamHI
PCR product
EcoRI
no stop
EcoRI
restriction digest
ligation
BamHI
B.
EcoRI
BamHI
EcoRI
pF1LZ
no stop
C.
G
V
H
pLCR
EcoRI
V seg
SalI
PCR product
D.
stop codon
EcoRI
SalI
EcoRI
pF1V3a
F1
V seg
EcoRI
PCR product
NdeI
F1
V seg
BamHI
E.
NruI
NdeI
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EcoRI
PstI
NruI
pET19b
pF1Vs
F1
V seg

REDACTED

RGURE 2

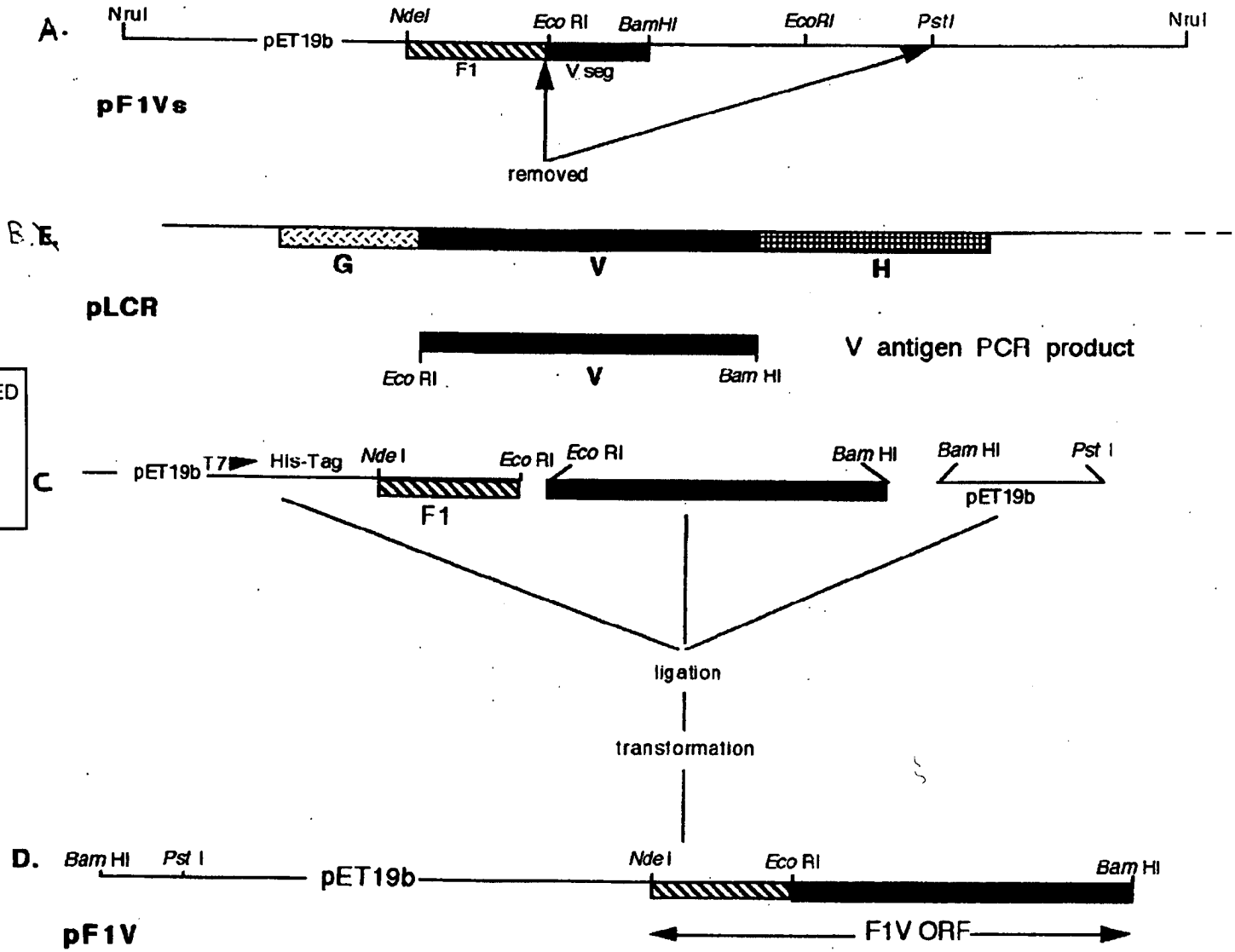


FIG. 3

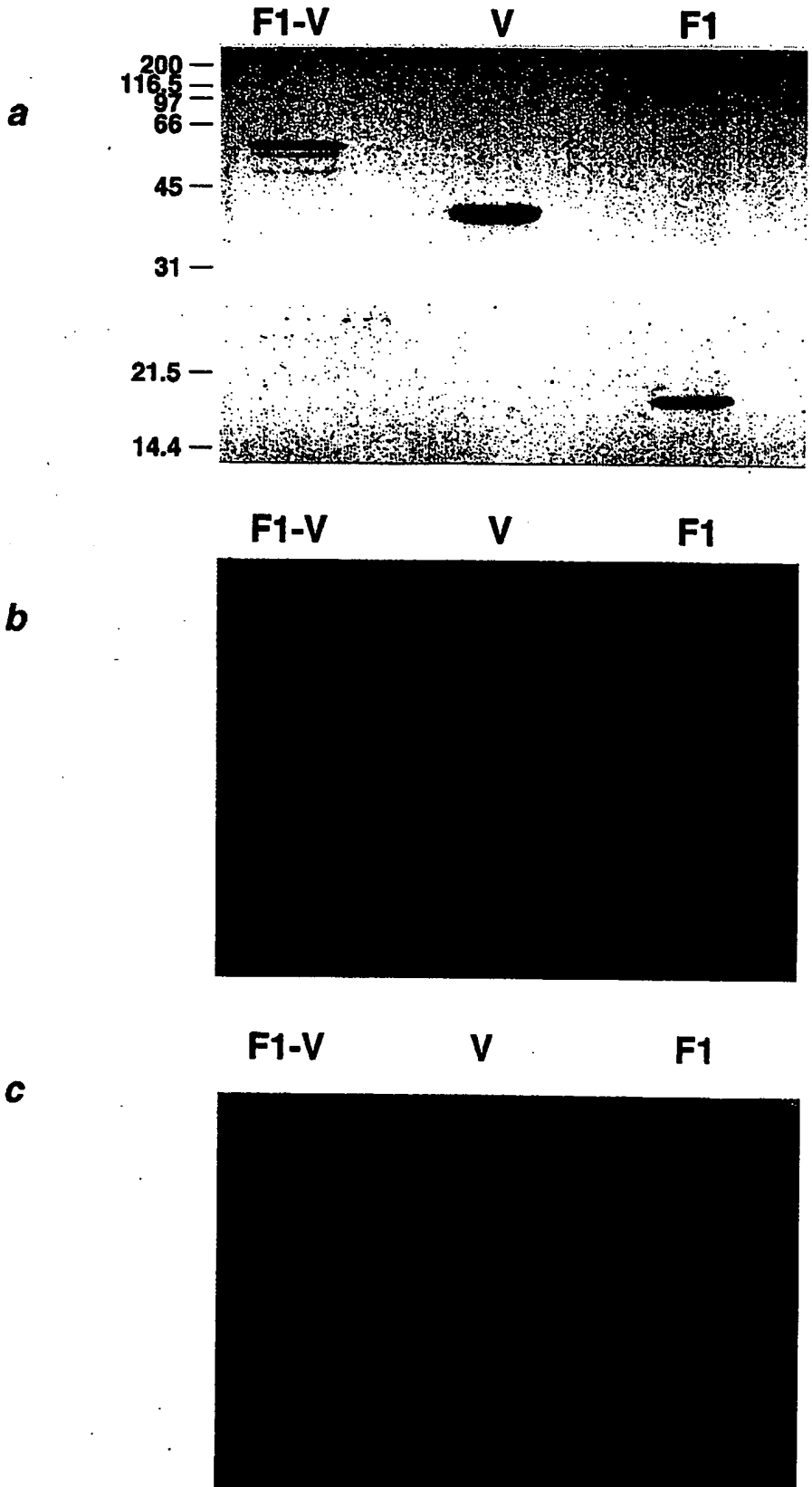


Exhibit DH4

REDACTED

Need to set up Western using Rabbit #1 & 2 & F1

will use 3ul of 30% cut from Run #2 as an F1 product to see if rabbit AB reacts / F1

will run a 14% gel from NOVEX & use pre-immune & & plug Antibody as a control

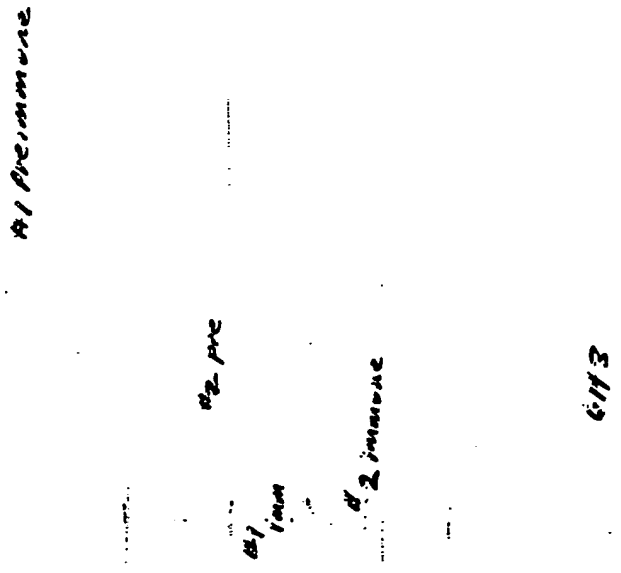
6c/#1

Lane	#1	2	3	4	5	6	7	8	9	10	11	12	13	14	1.
	MW #7		MW	F1		F1		F1		F1		#1			
	30%			30%		30%		30%		30%		30%			
Coomassie Stain 3ul + 3ul dye				Rabbit #1		Rabbit #2		Rabbit #1		Rabbit #2		& Plague			
				Pre-immune		Pre-immune		immune		immune		1/2000			
				1/2000		1/2000		1/2000		1/2000					
				3ul		3ul		3ul		3ul		3ul			
				3ul dye		3ul dye									

so add 25ul 30% cut to 25ul buffer - boil & add 6ul/lane - transferred ON to nitrocellulose

REDACTED

- did Western according to Gerry's procedure
- did 1/2000 for ea AB & 1/2000 / conjugate



REDACTED

Will ligate together different components of F & V as follows:
- Digest F1-Bam-Eco & Eco-Sal-V after LMP isolation
- F1-Bam-Eco & Eco-Sal-V will ligate for ~ 2 hr.
- then add B/Eco frag. from pYPR1 & pBluescript Eco/Sal

- Ran gel as follows: lane # 1 2 3 4 5
1% LMP 1KB R1 V(ARTIGUA) pBlV R1
B/E 275 E/Sal E/B → isolate small E/B frag. from pYPR1
E/Sal 165

Cut out of LMP the four bands F1 comp. B-Eco., Van. Eco/Sal 165 275 pBlue & Eco/Sal pYPR1 small Eco/Bam HI frag.

In 40 ul reaction ligated F1 comp. Bam/Eco PCR & Van. PCR Eco/Sal Sal. Sal01 2 ul 8 uls. Ulig to get a Bam/SalI frag.

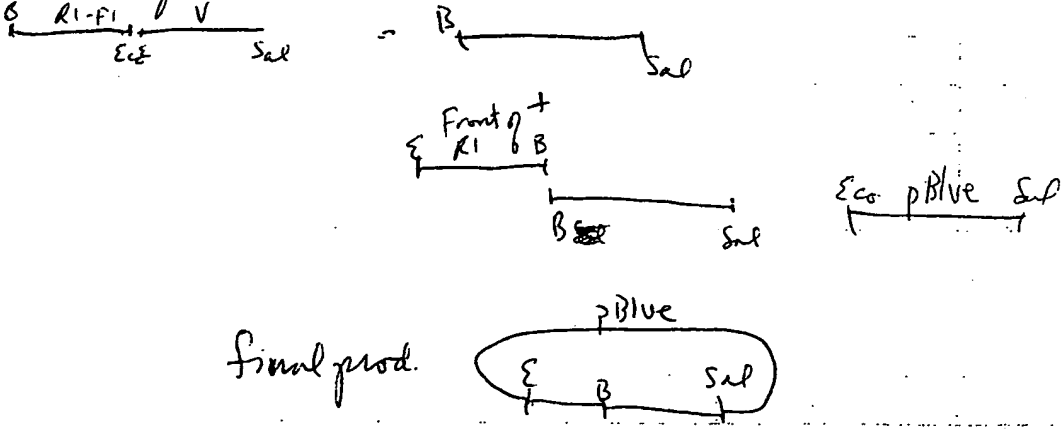
REDACTED

take Bam/SalI frag. ligation + added Sal pYPR1 E/B & Sal pBlue Eco/SalI frag. & 2 ul dH2O, 8 ul lig. Buffer + 2 ul ligase = 80 ul total
- ligated for 5 hr @ Rm temp.

- did transformation into DH5α competent (BRL) & 20 ul lig. mix / cells (100 ul) x 2

- plated 10 plates @ 100 ul / plate onto LA amp 100/Xgal

ligations as follows:



final prod.

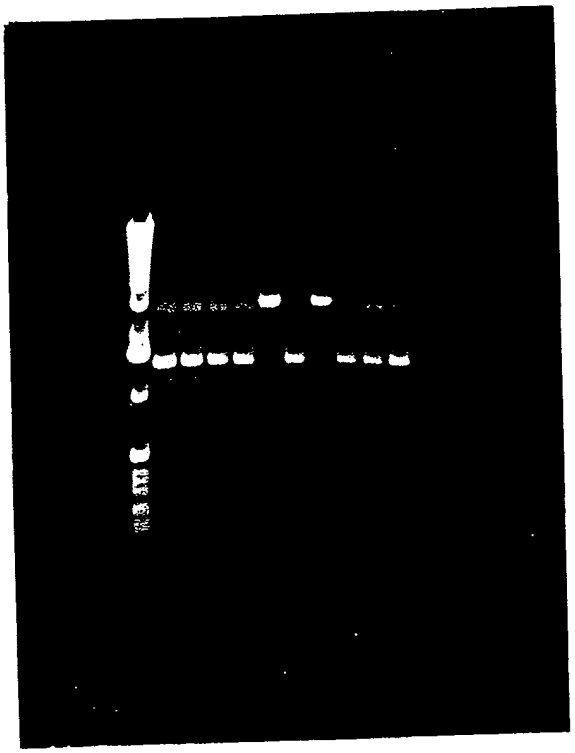
REDACTED

Got lots of white colonie from F1-V fusion in pBluescript. Will make rapid plasmid DNA of lots of clones

REDACTED

Made rapid plasmid DNA of 10 white colonies of F1-V ligation.

digested w EcoRI/Sal I & loaded to get 2.9KB, 7.9KB & .3KB. Another set of clones that were difficult to interpret but two clones the V segment ~~probably~~ inserted. #5 & #7 must have small EcoRI/Sal I of V inserted.



- Could now cut these plasmids w V to completion w BamHI & then EcoRI & ligate in the BamHI/EcoRI frag. of F1 complete into these clones. Then see if this resulting clone is cut internally w XbaI. If not clone in the small BamHI/EcoRI frag of pYPK1 by cutting w XbaI/BamHI & collecting the small X/B frag & ligating in the V gene clone.

→

Turbo Gopher

MEETING NOTES FROM SUE WELK

MTG NOTES WELKOS

REDACTED

Art

Exhibit DH5

IBC Stuff

1) Generic protocol for Pesh
Gerry A.'s w/ R memo

2) Ward at Navy: F1 crystallization (with a protective Ab),
Morrow at Navy – expression vector for V.
will send him the V clone (Heath's)

3) Brubaker / Saratov* group
b) protein Vs. 100 LD50 of WT plague, SC, done in Russia
using their V fusion.
Protein A
b) Making a V fusion w enterokinase site

Kutyrev* + Zilinov – same as Leppla's (Stepanov) friends → not able to get them to come.

Protein A – (Ig)H

V from Y. pth was probably

Brubaker hypothesis
V = colicin-like ..... not involved in Ca++ regulation
H = immunity

4) J. Burens @ made ~ 8 MAb to F1
will provide frozen seed myelomas.
" " Supernatants also.

Simpson's F1 clone (E.coli pVPR 1/5) ; system to screen MAbs using opsonic activity → Tobery's expt
Pretreat clone E.coli w/ F1
Put on MΦ +/- MAb and body cells
The E.coli F1 clone resists phagocytosis.
∴ See if the MAb allows phagocytosis

(b) Navy contract with P. Turnbull: To make stock cultures of diff. bact. + viral agents from around the world

5) Robert Ulrich
Naked DNA immunization: searching for an application.
LTR retroviral system

Uses rat cells
Provide him with Fl clone → (1) transfect directly ~~to~~ rat cells in vitro. Put cells sc into muscle.
(2) Inoculate animal directly with the DNA

Jerry A.
Preparative Super-Dex Column
2.5×10^6 mw
the stable
will exclude nFl aggregates - get in void volume.
Other contaminants are retained.

Acetate dry → NaCl extract → 30% NH_4SO_4 cut
→ +/- 25% cut to remove pH6 Ag → Superdex/void vol.

Dave Heath
Cloned the ~~ler~~VGH lcr GVH (2.1 kB) in pBLSkII (1) Sequenced the ends to prove was ok. (2) 2 internal V gene primers 168-275 aa aa } see Brubaker's preprint

made fusion of Fl and V, using this internal sequence

W. Simpson's: Eco RC A — Bam PCR dom — Fl caf — Eco → pBL SkII

Dave's Fl construction
dropped the TAA at end of Fl gene.
Added 6nt for EcoRI site.
Added GC clamp
Made primers to get a Bam-Eco fragment on PCR
aa v aa

(over)

Heart [REDACTED]

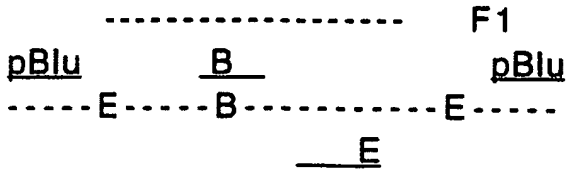
Exhibit DH6A

CREATION OF F1-V FUSION IN E. COLI

1. Initial part of project is to syntesize primers for the PCR of relevant segments of F1 and V. Will do PCR of F1 operon from the BamH1 site up to but not including the stop codon of F1.

BamH1 5' F1 primer: 5' GAA AAA GAA TCA GAG GAT CCT TTC 3'

EcoR1 F1 rev primer: 5' CTC GAA TTC TTG GTT AGA TAC GGT 3'



B E = F1 PCR product

Explanation of Primer creation:

BamH1

Beginning of F1 at BamH1 site: GAA AAA GAA TCA GAG GAT CCT TTC

GLU LYS GLU SER GLU ASP PRO PHE

End of F1 = ACC GTA TCT AAC CAA TAA

THR VAL SER ASN GLN

Primer creation at end of F1:

5' ACC GTA TCT AAC CAA 3' (no TAA included)
3' TGG CAT AGA TTG GTT 3' compliment

5' TTG GTT AGA TAC GGT 3' reverse compliment

EcoR1

add EcoR1 site and GC clamp: 5' CTC GAA TTC TTG GTT AGA TAC GGT 3'

Final F1 PCR product after restriction:

BamH1 EcoR1
GAT CCT TTC---ACC GTA TCT AAC CAA G
GA AAG---THR VAL SER ASN GLN CTT AA = 3.6 kb

SYNTHESIS OF INTERNAL V FRAGMENT:

1. Portion of V to be added to F1 is from a.a. 168 to 275, so this is a 321bp segment encoding for 107 a.a.

168: TCA GTT ATT CAA GCC GAA ATT AAT---ACC ACC TGC TCG GAT :275
SER VAL ILE GLN ALA GLU ILE ASN THR THR CYS SER ASP

Primer design:

EcoR1
Vfor168-275: 5' CAC GAA TTC TCA GTT ATT CAA GCC G 3'
SER VAL ILE GLN ALA

Vrev168-275: 5' ACC ACC TGC TCG GAT TGA 3'
THR THR CYS SER ASP STOP
3' TGG TGG ACG AGC CTA ACT 5' compliment

5' TCA ATC CGA GCA GGT GGT 3' reverse compliment
Sal1
5' GTGG GTC GAC TCA ATC CGA GCA GGT GGT 3' rev. compliment with Sal1

Final PCR product of V168-275 after digestion: stop codon
EcoR1 Sal1
5' AA TTC TCA GTT ATT CAA GCC----ACC ACC TGC TCG GAT TGA G
G AGT CAA TAA GTT CGG TGG TGG ACG AGCCTA ACT CAGCT

Final PCR ligation of F1 and V168-275:

F1 product from BamH1 to end of F1 ORF:

BamH1 EcoR1

GAT CCT TTC ---ACC GTA TCT AAC CAA G

GA AAG---THR VAL SER ASN GLN CTT AA

Begining of V168-275:

EcoR1

AA TTC TCA GTT ATT CAA--- Sal

G SER VAL ILE GLN

Results after ligation of F1 to V168-275:

BamH1 EcoR1

GAT CCT TTC----ACC GTA TCT AAC CAA GAA TTC TCA GTT ATT CAA---Sal

GA AAG THR VAL SER ASN GLN GLU PHE SER VAL ILE GLN

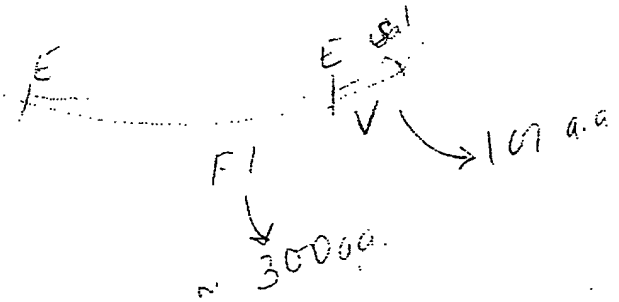
To this ligation product, ligate the small Sst1/BamH1 of pYPR1 so that the complete F1 operon is back together and now ligate this product to an Sst/Sal1 digested pBluescript. Total insert size should be 8.2kb.

Final Construct

pBluescript

TOTAL = 11 KB

Recombinant is sticky; put insert into pBS 32.2

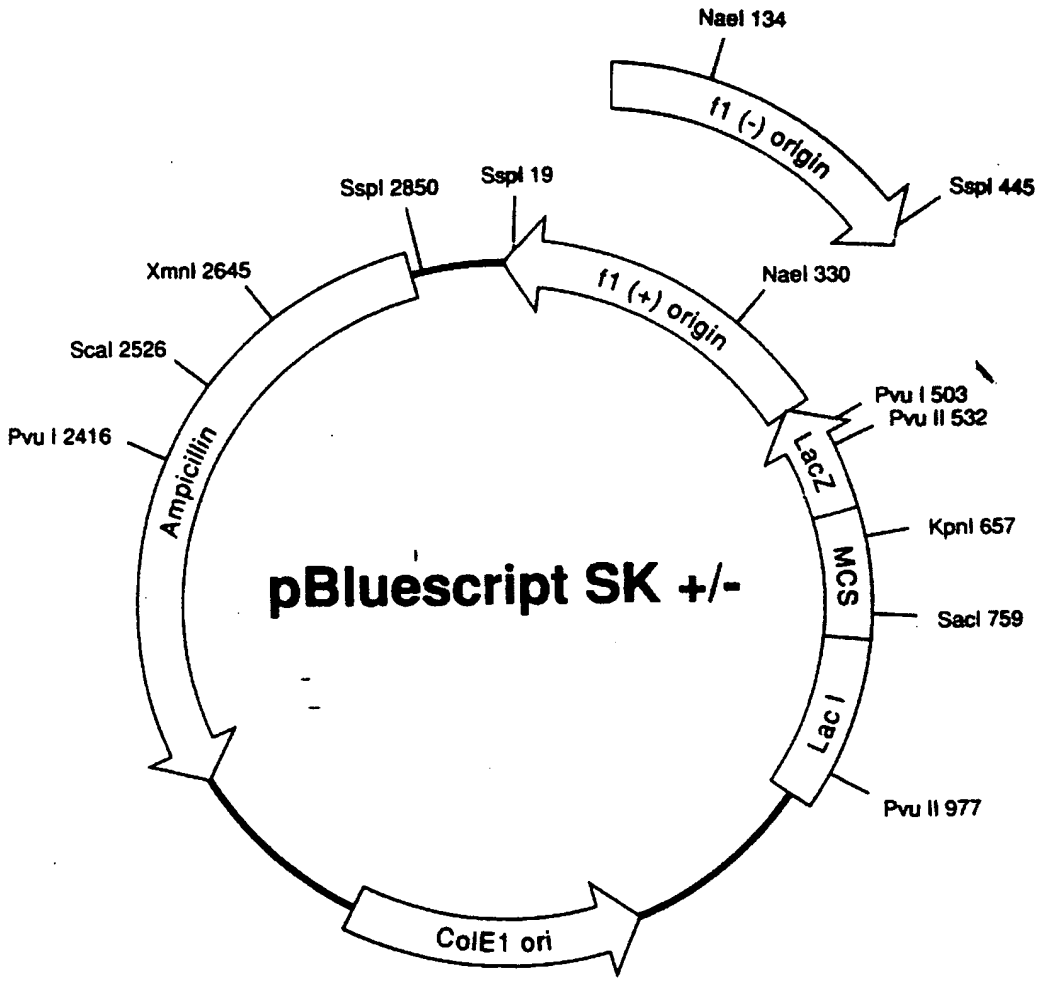


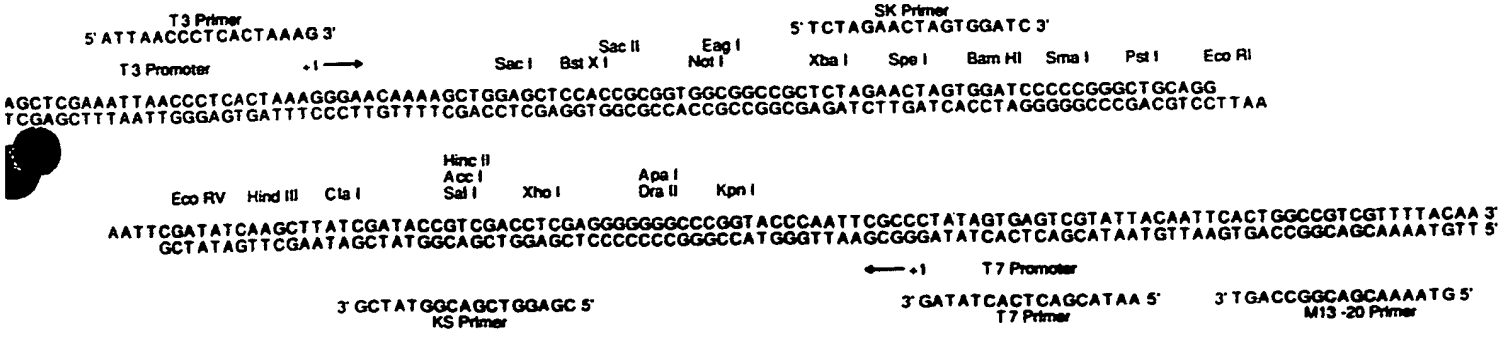
Wednesday, REDACTED 3:46 PM

Sequence Range: 1 to 193

```
     5    10    15    20    25    30    35    40    45    50    55    60
     *     *     *     *     *     *     *     *     *     *     *     *
MKKISSVIAI ALFGTIATAN AADLTASTTA TATLVEPARI TLTYKEGAPI TIMDNGNIDT

    65    70    75    80    85    90    95   100   105   110   115   120
     *     *     *     *     *     *     *     *     *     *     *     *
ELLVGTLTLG GYKTGTTSTS VNFTDAAGDP MYLTFTSQDG NNHQFTTKVI GKDSRDFDIS

   125   130   135   140   145   150   155   160   165   170   175   180
     *     *     *     *     *     *     *     *     *     *     *     *
PKVNGENLVG DDVVLATGSQ DFFVRSIGSK GGKLAAGKYT DAVTVTVSNQ EFDIKLIDTV

   185   190
     *     *
DLEGGPGTQF AL*
```

EcoR I
polylinker

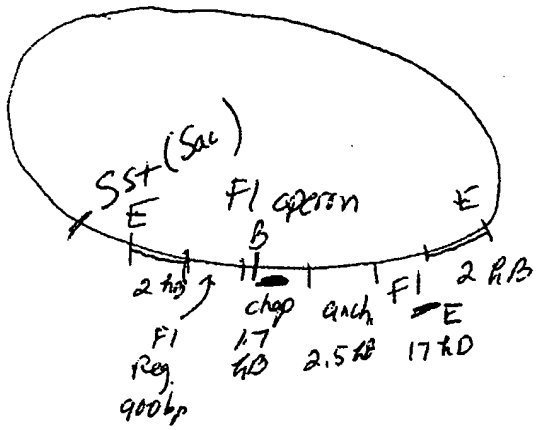




REDACTED Staff meeting : DAVE HEATH

Bubaher's - found immunogenic regions of V
= 168 - 275
fusion to a protein vector & deletion analysis

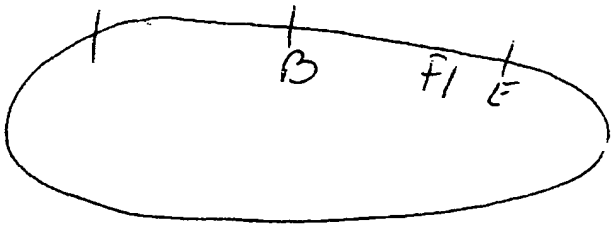
Dave fused this segment onto end of F1 gene



9.4 kb EcoRI fragment
need entire operon to get F1 expression (+ extend capsule mode in E. coli)

Bam-EcoRI subclone : get no F1 expressed.
Using a primer w/ EcoRI site,
"fused EcoRI site next to last stop codon of F1 structure.
Took out Bam-Eco frag

∴ has entire operon w/ F1 with no stop codon!



reads 22 amino acids until runs into stop codon in F1

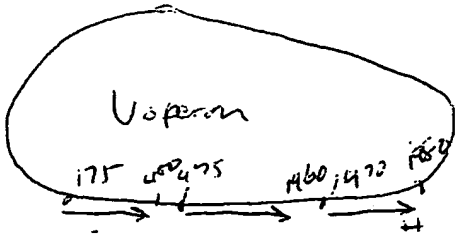
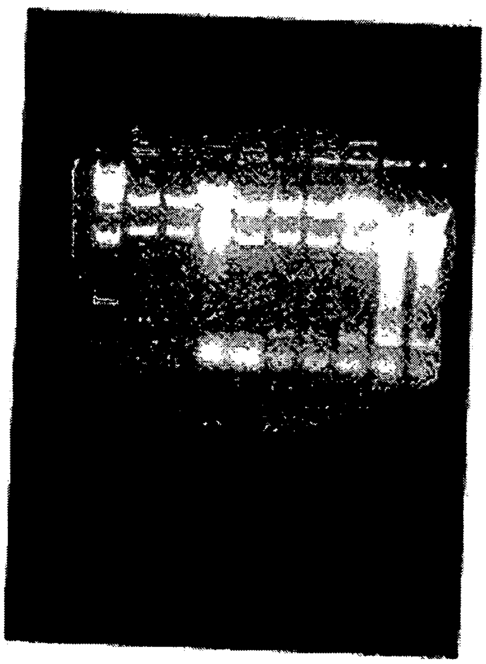


Exhibit DH7A

REDACTED

Made rapid plasmid preps of candidates that may have the ... and $V_{151-235}$ fused. Calling them pFIV2 & pFIV3. ... analysis (page 55?) says pFIV3 is in good shape & fused.

Lane # 1 2 3 4 5 6 7 8 9 10
λ ... pFIV3 a b c d E
HindIII



Results: all five pFIV3 a-E preps have small V segment when digested w/ E/B ← See gel

Grew up 5ml LB/Amp ... cultures in 250 ml flasks in shaking water bath

HB101 ———————

pVR1 pBlue pFIV2 pFIV3a & B

Will make a pellet & supernatant sample of each BN for SDS-PAGE & Western Blot.

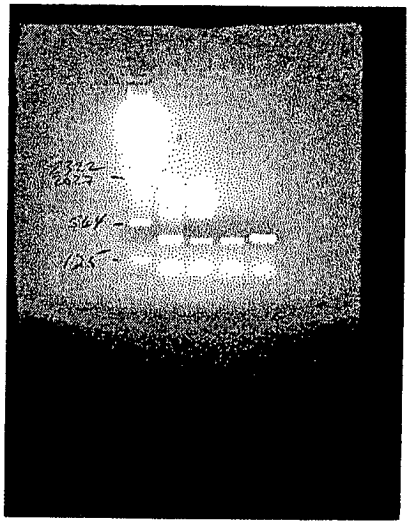
REDACTED

- All 5 cultures grew but pFIV3B grew poorly. Will collect a separate supernatant & pellet sample at the end of the day.

REDACTED

PCR to see if F1 + V are fused. Used F1 forward primer and V reverse primer. Did 4 samples. #2, #3, #4 + pF1-LV3 DNAs

lane # 1 2 3 4 5
MW #2 #3 #4 pF1-LV3
λ
HindIII



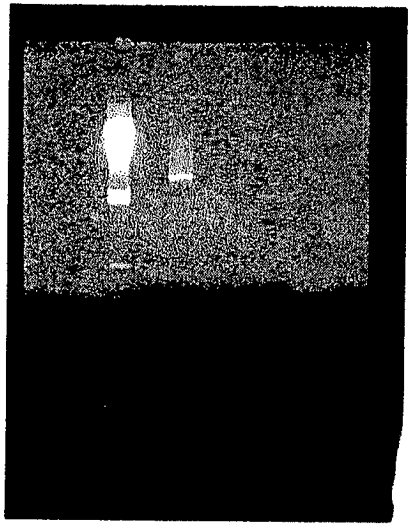


Exhibit DH7B

Exhibit DH8

Gerry's colleague is looking for other regulators in Y. pestis (4th Katherine

Global thermoregulator

REDACTED

1. Dave's fusion of V-F1 : need good monoclonal α V
AF will ask Brubaker.
Also to get the monoclonal of Karu's Mab, from FDA

2. Abstract & Publication - group ~~endeavor~~ endeavor / authorship!

3. F1 purification + preps
① 32 mg of supernatant F1 - Andrew
② Dave's prep

Cutter F1 looks diff. on gel than Gerry's extract

4. Mice to test our F1 preps:

1) Parenteral challenge Amice w. 100 LD50's:
- 4 diff. doses of the F1 preps, (2 doses of each prep)
- Cutter vaccine control: and F1 extracted from Cutter vaccine
- Aluminum hydroxide adjuvant

NEED
1. F1 ELISA:
Cindy Ross / D. Maughio
?
Using F1 from W. Reed prep

F1 capture ELISA ..... Applied Research says have developed one.
Koch says can detect ≥ 5 ng F1/ml

George Anderson:
Chris Bolt setting up to do Ezell's α F1 Elisa
Navy has an F1 fiberoptic detection system developed → F1 in serum detected.

5. One of the monkeys receiving 17 CFU had an IgM anti-F1 response. To check it: Baseline serums for reactivity to F1

f

b, EM - Terry A. can do regular negative staining:
drop unfixed bacteria on slide / formvar-coated grids

↳ Status of bacterial pellets (work / gold labeling?

1. Wareham's protocol to put mutator back into X. pestis (IBC - 12/8
sacB gene = a new Bacillus gene --- must get full
RAC review:
Any time put DNA of foreign origin into X. pestis (class 3)
must get RAC approval.

Exhibit DH9

REDACTED

Ran 14% PAGE Gel of V to test antisera from my rabbit.
Rabbit # ~~7F7D771F4A~~
7F7D23C62c

Gel #1

1	2	3	4	5	6	7	8	9	10
MW	F1-V	F1pure	V antigen	pblue	MW	F1-V	F1-pure	V ant.	pblue
10ul	20ul	3ul	10ul	10ul					

Gel #2 = same as #1

- transfered both gels to nitrocellulose and divided into 4 segments after transfer, each segment being identical

- 1st seg. = αPAV 1/2000
- 2nd " = αPAV 1/10,000 } my rabbit
- 3rd " = αPAV 1/20,000
4 seg = αPAV 1/100,000 = Brubaker's serum.

REDACTED Developed ~~gel~~ westerns (opposite page) & found my rabbit αPAV reacted c̄ my F1-V fusion protein & best dilution appears to be 1/10,000.

REDACTED - stripped the same blots & blocked c̄ Blotto. Then added my rabbit αF1 (E. coli absorbed) @ 1/2,000, 1/10,000 & 1/20,000. Came back c̄ goat α Rabbit HRP & developed.

Result: In all

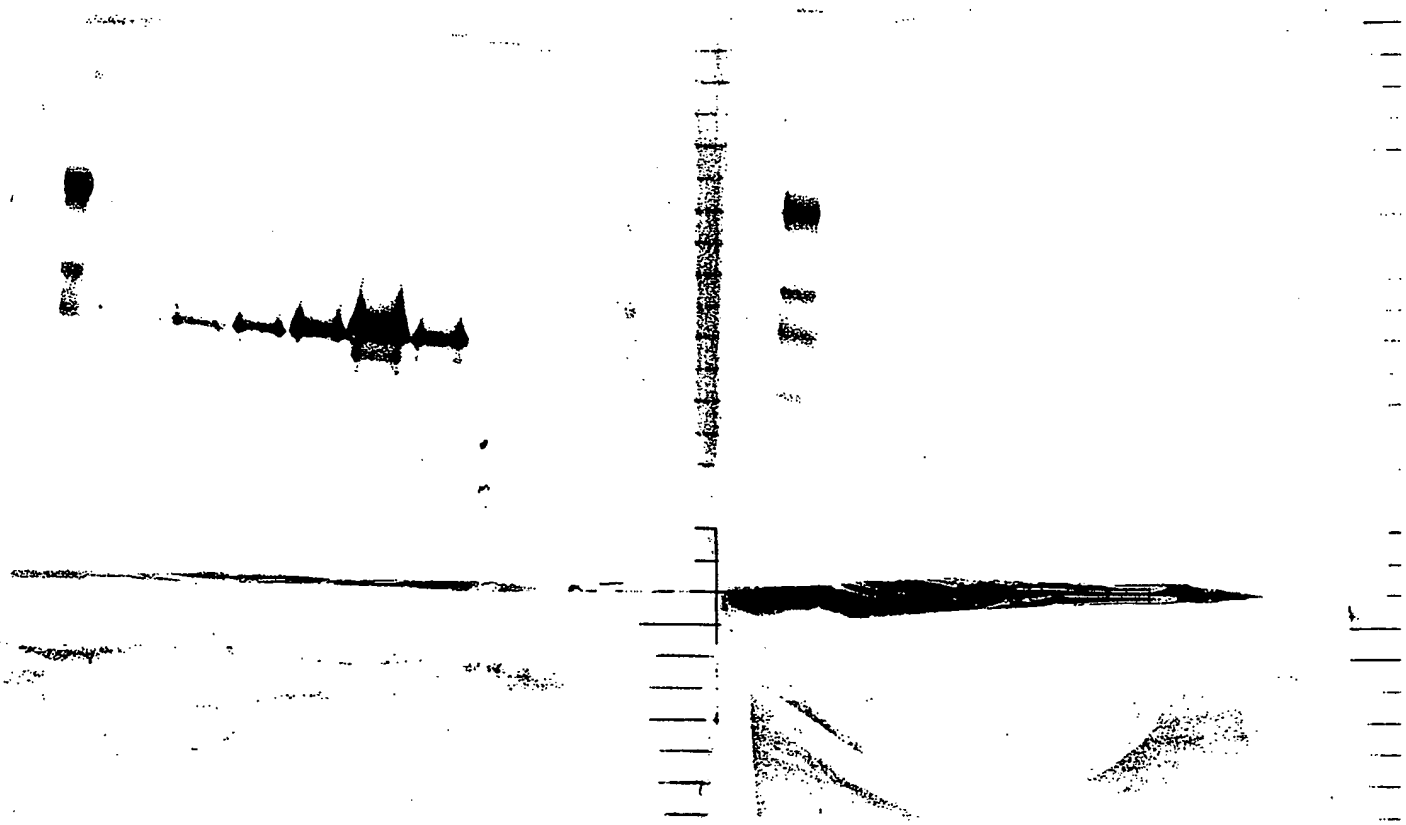
1) Brubakers αPAV reacts c̄ my F1-V fusion protein
2) my Rabbit αPAV needs boosting but does react c̄ Brubakers PAV & my F1-V fusion protein
3) The F1-V used here was extracted from capsule coming from pF1V3a in E. coli pR745
4) The rabbit αF1 that I made reacts c̄ pure F1 & the extracted F1-V fusion protein but not c̄ V alone. Therefore, the F1-V fusion does contain epitopes specific for F1 & for V.

REDACTED

Also did a 3rd purification of FIV on Ni^{2+} column. Did as before also ran all fractions on a 4-20% Tris-Gl Novex Gel. Also time also ran separate gels to see what ca off of the immidizole wash @ 20mM.

Gel #1 = 300mM immidizole wash

8 7 6 5 4 3 2 frac't. Frac 13 12 11 10 9



Gel #2 20mM immidizole

Exhibit DH11

FIV as a vaccine against F1+ & F1- plague

REDACTED

Purpose: use of FIV as a vaccine against F1+ Y. pestis and F1- Y. pestis challenge in mice.

Calculations concerning FIV exp. by group

1st WEEK wet to ē alhydrogel

Group 1 = control = dilute alhydrogel 1/7 in PBS

Group 2 + 6 F1 capsule extract

take 350 ug F1 + absorb to 1 mL alhydrogel (ON @ 4°C

dilute 1/7 = 50 ug/mL

Groups 3 + 7 take 2mL F1 capsule extract (.530 mg/mL) and treat as follows:

add 135 ul (4x ELVIS in BUFFER - NOVAGEN) to 1mL F1 extract + mix

" 0.72 g Urea + mix

" enough F1 extract to equal 2mL

- let sit on ice for ~ 4 HRs

- then dialyze vs. 3M Urea ē 2 changes (1 ON) (1 liter ea)

- dialyze vs 1M Urea in PBS ē 2 changes (1 liter ea)

dialyze vs. PBS alone vs. 2 changes - check conc. on BCA

- then absorb 350 ug to alhydrogel (1mL) + dilute 1/7 to = 50 ug/mL

Group 4 + 8 FIV absorption to give 18.5 ug/mouse x 10 mice = 185 ug

= 92.5 ug/mL for 2mL

92.5 x 7 = 647.5 ug

so take 647.5 ug FIV + add to alhydrogel + absorb ON @ 4°C

Group 9 FIV absorbed to alhydrogel at 37 ug/mouse for 10 mice

= 185 ug/mL x 2 mL x 7 = 1.295 mg

1.295 mg + add to alhydrogel = absorb + dilute 1/7

Creation of F1 fused to the entire ORF of V antigen.

1) Made a qiagen prep of petF1V1A3-2 and restriction with EcoR1 and BamH1. - gets F1 gene out

2) Did a PCR reaction using of a whole plasmid prep (Qiagen pure DNA) from both *Y. pestis* antigua and pestoides as template DNA. The PCR reaction was as follows:

10 ul MgCL2 buffer
05 ul Template DNA
08 ul 4 nucleotides
02 ul forward primer*
02 ul reverse primer*
72.5 ul dH2O
0.5 ul Tac Polymerase

added 2 drops of mineral oil and placed in PCR machine. Did 30 cycles at 94 C, 1 min; 55 C, 1 min; 72 C, 2 min; then 72 C for 6 min. followed by 4 C overnight.

3. Ran PCR reactions on LMP agarose and sliced out a 1 Kb band and heated at 68 C and removed 5 ul into a 20 ul total enzyme digest with *EcoR1* and *BamH1.* Allowed digestion overnight at 37 C. Heated reaction at 68 C for twenty minutes to destroy enzyme activity.

4. Ran out on LMP agarose the digest from #1 above and isolated the approx. 5 kb band. Heated at 68 C along with LMP PCR product using antigua PCR DNA and placed in a ligation reaction as follows:

4 ul ligase buffer
7 ul dH20
5 ul PCR product (V antigen)
3 ul vector (pET19BF1V1a E/B)
1 ul ligase

5. Allowed reaction mix to ligate overnight at 4 C.

6. Electroporated E. coli BLR (Novagen) with 5 ul of the ligation mix. Plated cells on LB/Carbenicillin 100.

*Forward primer = 5' CGC GAA TTC ATG ATT AGA GCC TAC GAA 3' pevc
(ForEcoV)
*Reverse primer = 5' CGC GGA TCC TCA TTT ACC AGA CGT GTC A 3' pbve
(RevBamV3'end)
14 APr. 95

After several unsuccessful attempts to ligate the entire V sequence to the end of F1 at the EcoR1 site, I restricted pF1V1a 3-2 with EcoR1 only and found 2 cut sites rather than the single site I thought was there. This could account for the inability to clone V into pF1V1a 3-2. I therefore decided to take pF1V1a 3-2 and perform an additional PCR using the forward T7 promoter primer and the BamH1V(275) primer to make a new PCR product. This PCR product should be resticted with NcoI and EcoR1. I can then ligate to the EcoR1/BamH1 V entire PCR product for ligation into an NcoI/BamH1 digested pET19B. Experimental outline follows:

This gives F1 gene

PCR: 5 ul pF1V1a 3-2
2 ul T7 primer
2 ul BamH1revV275 primer
8 ul Nuc's
10 ul PCR buffer
72.5 ul dH20
0.5 ul Tac polymerase
30 cycles; 94, 1 min; 55, 1 min; 72, 2 min; then 5 min at 72.

This will generate F1 gene after restriction ~ Nco & EcoRI

Will gel extract the PCR product using the Qiagen gel extraction kit and then restrict with NcoI/EcoR1 and run fragment through LMP agarose for purification. Will Hoverson is plasmid purifying the V segment cloned into pBluescript and I with gel purify the V EcoR1/BamH1 insert DNA by running it on Low Melt agarose. Final ligation will look something like this:

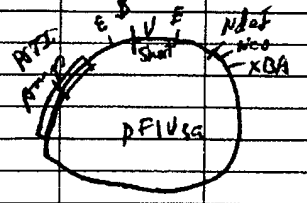
3 ul pet19b (LMP purified) resticted with NcoI/BamH1
3 ul Nco1/EcoR1 F1 insert (LMP purified)
3 ul V antigen restricted with EcoR1/BamH1
4 ul ligation buffer
1 ul ligase
6 ul dH20

~1.1 Nco/ pci

I will then electroporate *E. coli* BLR with the above ligation mix and look for colonies which contain a 1 Kb EcoR1/BamH1 fragment and a ~ 500 bp NcoI/EcoR1 fragment signifying that both F1 and V are together and attached. Will then examine colonies for expression of a fused F1 V protein by induction with IPTG followed by Western blotting using antibody specific for V and for F1.

REDACTED

After several attempts of ligating E/B of V antigen to pFIVsg E/B, I realized that there is an extra Eco site in PET19B. I came up with a new strategy for cloning the pFIV entire fusion:



i) cut pFIVsg ē Eco & PstI & isolate large Eco/PstI frag

ii) cut V entire ē BamHI & Eco

iii) cut PET19B ē pSTI & BAMHI & isolate small fragment

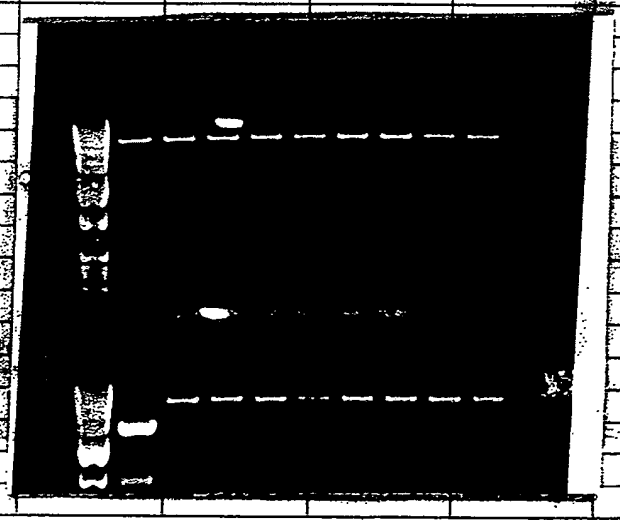
for ligation: add 3ul pFIVsg Eco/PstI large frag.
3ul V entire B/E
~~9~~ul PET19B ~~small~~ PstI/BamHI small frag
4ul ligase Buffer
1ul ligase
20

- place @ 4°C ON

REDACTED transform E. coli HB101 ē ligation mix

REDACTED - picked 18 transformants for growth in 2B/Amp50

REDACTED - made rapid plasmid DNA of the 18 transformants - see gel - & cut ē E/BAm - should see 1KB V fragment



Results: 1KB V fragment is present in several of the rapid plasmid preps. Will take DNA's from #2, #3 & #5 to electroporate BLR.

REDACTED

Results of electroporation were that #3 only gave transformants. Took 4 individual colonies from these transformants & grew separately ON in Amp^{50}

REDACTED

Had four separate flasks i ON growth labeled BLR (pFIVe) 3-1, 3-2, 3-3, & 3-4. Spun down the ON & resus. in fresh LB/CARB^{100} Grew each culture in 250 mL flask @ 5mL LB. Took OD readings @ ~ 2hrs. Had flasks for uninduced & induced for addition of IPTG -

		O.D. 600
3-1	uninduced	0.220
"	induced	0.221
3-2	uninduced	0.316
"	induced	0.334
3-3	uninduced	0.263
"	induced	0.229
3-4	uninduced	0.234
	induced	0.265

- at this time added IPTG to 1mM. Placed in shaker @ 37°C for 2 hours.

Final O.D. 600

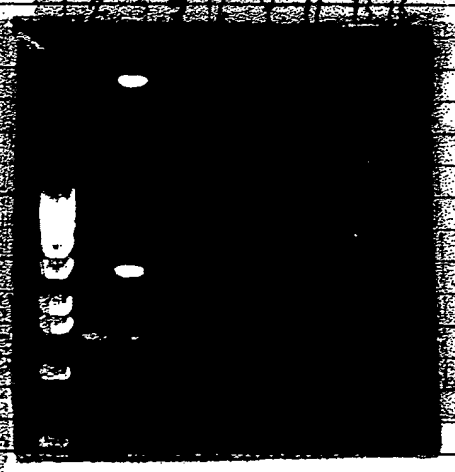
		ratios
3-1 un	1.78	> 1:3
3-1 ind	0.60	
3-2 un	2.24	> 1:3
3-2 ind	0.82	
3-3 un	1.96	> 1:4
3-3 ind	0.56	
3-4 un	1.86	> 1:3
3-4 ind	0.65	

Took 1mL of each induced culture & centrifuged. Resus. 3-1, 3-2, & 3-4 in 330 uL. for 3-3 resus. to 250

REDACTED

Did PCR of plasmid preps from previous page using #1 beginning primer (Nde-7 primer) & U Kan Hs end primer. Should get a 1565 bp prod. & do for almost all isolates.

On Coomassie gel #3 above didn't appear PI-Ventire. Must look at other preps.

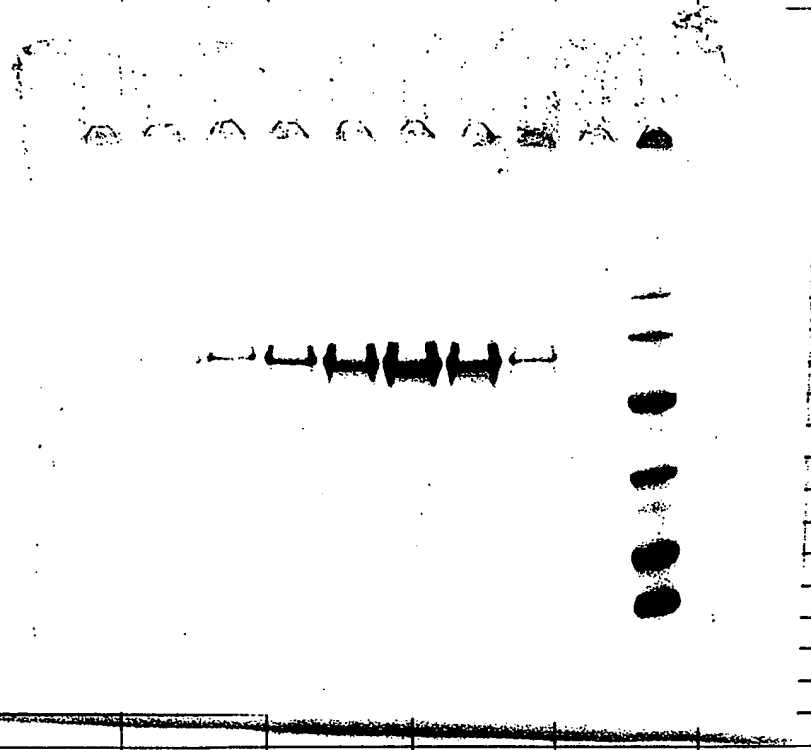


REDACTED

Gel of FPLC purification, using Ni++ column, for purification of FIVe.

Fract#

37 35 33 31 29 27 25 23 21



Pooled fractions 23-45 & did buffer exchange in centricons w/ 1X binding buffer in 6 M Urea

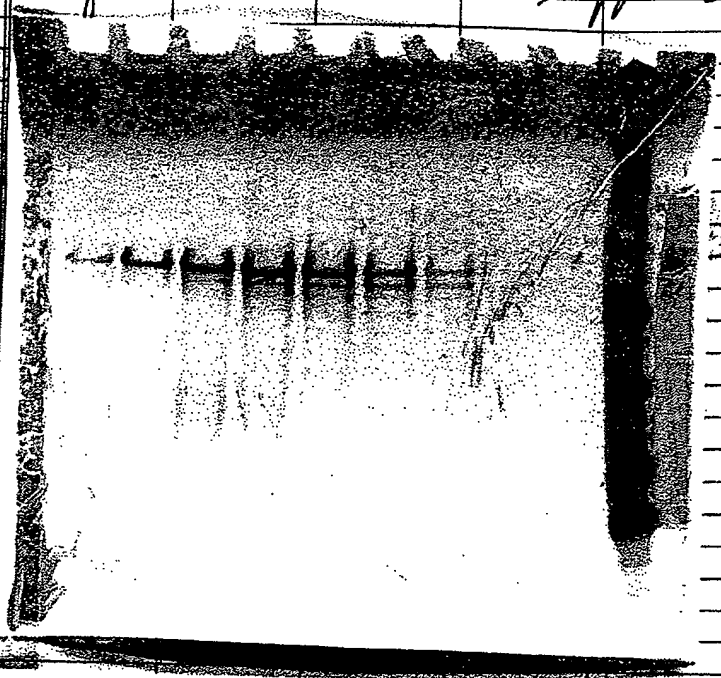
REDACTED Ran 4mL of total 8mL of 1st FPLC pool thru FPLC Again. Collected 45 fractions using method #7

REDACTED Ran 2nd 4mL thru FPLC & collected 45 fractions as for method #7. This will be the 2nd time thru the nickel column for this FIVectin batch from #7b.

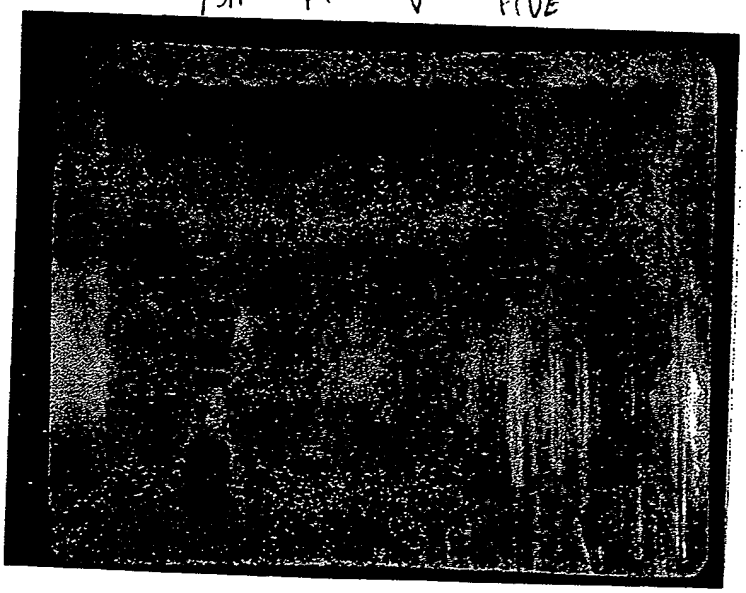
REDACTED - Ran 2, 10% Tricine gels of 2nd FPLC purification of FIVe. See following page

REDACTED

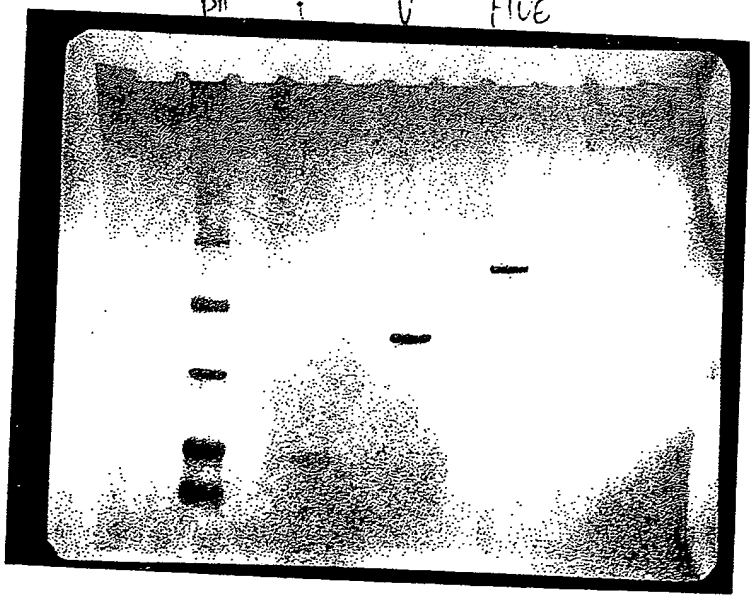
Ran another aliquot of FIVE thru the FPLC & checked fractions 21-37 for FIVE. Saw FIVE in 23-37. Pooled fractions 25-39 & buffer exchanged using centricon



P311 F1 V FIVE



P311 F V FIVE



Ran 10% Tricine of F1, V & FIVc

Exhibit DH16

Suggested protocol for the F1-whole V fusion protein.

File: F1-wholeV fusion last update REDACTED
Protocol: B95-01
F1-WV fusion protein immunization and challenge

Investigators: CPT Heath, Dr. Welkos, LTC Anderson, COL Friedlander

Background. CPT David Heath produced and purified a recombinant F1-V fusion protein. The protein is positive by Western blot to F1 and V. Only part of the V-protein was used in the intial F1-V fusion. This F1-V fusion was immunogenic, but was not very efficacious when compared to the whole V-protein (WV) produced by Mauro to protect against F1 minus strains of *Y. pestis*. This is a repeat of part of the intital F1-V protection study using the whole V-protein in the F1-WV fusion. Subcutaneous injection along back of the mouse for immunization.

Purpose: Immunize and challenge mice to check on immunogenicity and protection against the CO92 and C12 strain of Y. pestis by sc and aerosol challenge, **100-Max LD_{50}**.

Alhydrogel, 1.3%, from SuperFos. Batch # 2043, Expiration date None, _____µg of AL/dose

Endotoxin level in the F1-WV preparation is _____ U/ml.

Will use **Mauro's V** which has been urea treated as per the F1-WV procedure. Details in CPT Heath's laboratory notebook.

Immunization Groups: 10 Swiss Webster female mice per group from Harlan Sprague Dawley

Implantable Micro Identification transponders from: BioMeic Data Systems, Inc 255 W. Spring Valley Ave. Maywod, NJ 07607, 1-800-526-BMDS

Subcutaneous challenge		Strain	Dose LD_{50}	# Mice
Group 1	Alhydrogel alone, days 0, 30, sc	C12	100	10
Group 2	Alhydrogel + 13.6 µg F1-WV fusion protein day 0, 30, sc	C12	100	10
Group 3	Alhydrogel + 10 µg Mauro-V urea, days 0, 30,sc	C12	Max	10
Group 4	Alhydrogel + 13.6 µg F-1WV fusion protein days 0,30,sc	C12	Max	10
Group 5	Alhydrogel + 27.2 µg F1-WV fusion protein days 0, 30, sc	C12	Max	10
Group 6	Alhydrogel alone days 0, 30, sc	C12	Max	10
Group 7	Alhydrogel + 13.6 µg F1-WV fusion protein day 0, 30, sc	CO92	100	10
Group 8	Alhydrogel alone, days 0, 30, sc	CO92	100	10
Aerosol challenge				
Group 09	Alhydrogel alone, days 0, 30, sc	C12	50	10
Group 10	Alhydrogel + 13.6 µg F1-WV fusion protein day 0, 30, sc	C12	50	10
Group 11	Alhydrogel + 10 µg Mauro-V urea, days 0, 30, sc	C12	Max	10
Group 12	Alhydrogel + 13.6 µg F1-WV fusion protein, days 0, 30, sc	C12	Max	10
Group 13	Alhydrogel + 27.2 µg F1-WV fusion protein days 0,30, sc	C12	Max	10
Group 14	Alhydrogel alone, days 0, 30, sc	C12	Max	10
Group 15	Alhydrogel + 13.6 µg F1-WV fusion protein days 0, 30, sc	CO92	100	10
Group 16	Alhydrogel alone days 0, 30, sc	CO92	100	10
~~Group 17~~	~~Alhydrogel + 13.6 µg F1-WV fast prep, days 0, 30, sc~~	~~C12~~	~~Max~~	~~10~~
Group 18	Alhydrogel + 10 µg F1 + 10 µg Mauro's V, days 0, 30, sc	C12	Max	10
Group 19	Greer plague vaccine, days 0, 30, sc	C12	Max	10 05
Group 20	Alhydrogel alone, day 0, 30, sc	C12	Max	05

180

Group 21	ALH + 13.6 µg F1-WV fusion protein day 0, 30, sc --antibody response Measure titer at 7,14, 27,57, 90		10
~~Group 22~~	~~ALH + 13.6 µg F1-WV fusion protein day 0, 30, sc --lung lavage, day 57~~	~~x~~	~~05~~
Group 23	ALH + 27.2 µg F1-WV fusion protein day 0, 30, sc --antibody response Measure titer at 7, 14, 27, 57, 90		10
~~Group 24~~	~~ALH + 27.2 µg F1-WV fusion protein day 0, 30, sc --lung lavage, day 57~~	x	05
~~Group 25~~	~~ALH + Mauro-V urea, 10 ug, day 0, 30, sc --antibody response~~		~~10~~
~~Group 26~~	ALH + Mauro-V urea, 10 ug, day 0, 30, sc --lung lavage, day 57	x	~~05~~
~~Group 27~~	~~ALH alone, day 0,30, sc~~ ~~Measure titer at 7, 14, 27,57, 90~~		10
~~Group 28~~	~~ALH alone, day 0,30, sc, --lung lavage, day 57~~	x	05
Group29	ALH alone, for spleen weights 28 day pi		~~10~~ 05
		Total	220

Schedule

Groups 1-20

13Jun95	Arrival of Swiss Webster mice, female 7-8 wks, Harlan Sprague Dawley in AA-3 (Barrier)
24Jun95	Chipped with BioMedic Data Systems transponders, West
27Jun95	1st immunization, day 0
27Jul95	2nd Immunization, day 30
24Aug95	Bleed to determine prechallenge titers, day 58
31Aug95	Challenge by aerosol & sc routes, day 65
28Sep95	Terminal bleed, day 28 pi, titrate spleens# serum #

Group 21-25

13Jun95	Mice arrive
27Jun95	1st immunization, AA-3
11Jul95	Groups 21, 23, 25; Bleed, day 14, AA-3, SERUM#
26Jul95	Groups 21, 23, 25; Bleed, day 29, AA-3, SERUM#
27Jul95	2nd immunization, day
31Aug95	Bleed, day 65, AA-3, Groups SERUM# Groups 22, 24, 26, and 28 for serum titer & bronchial lavage #
28Sep95	Group 29 for Spleen weights #
25Sep95	Groups 21, 23, and 25; day 90, serum #

Chip numbers for all groups extra alhydrogel controls

Sequence Range: 1 to 1566

```
                                                                      60
                         *                   *                    *
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                                                                     120
                  Cl Sal HS *                *                    *
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                                                                     180
                         *                   *                    *
ACTGCTAATGCGGCAGATTTAACTGCAAGC ACCACTGCAACGGCAACTCTTGTTGAACCA

                                                                     240
                         *                   *                    *
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                                                                     300
                         *                   *                    *
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                                                                     360
                         *                   *                    *
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                                                                     420
                         *                   *                    *
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                                                                     480
                         *                   *                    *
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                                                                     540
                         *                   *                    *
GGCAGCCAGGATTTCTTTGTTCGCTCAATT GGTTCCAAAGGCGGTAAACTTGCAGCAGGT

                                                                     600
                         *                  *EcoRI                *
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                                                    M  I  R  A  Y>
                                                  ___GENE=LCR____>

                                                                     660
                         *                   *                    *
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AAATGA
 K  *>
_____>
```

REDACTED

SET UP ABSORPTIONS FOR LTC ANDERSON:

FOR GRPS 000A + 000B used FI-V that had been purified by Ni++ & run through a Sartorius Q15 filter using 10 mM Tris, pH 7.6 0.5 mM EDTA + 0.5 M NaCl for elution. Buffer exchanged this using 10 mM Tris, pH 7.6 + 0.5 mM EDTA using a centricon. Did BCA assay for protein conc. = 690 ug/mL and 1.5 mL total volume

so used 450 ug in 3 mL PBS = 652 ul of FIV (@ 690 ug/mL)

652 ul FI-V + 2.348 mL PBS

FOR GRPS 00A 00B same FI-V as above @ 690 ug/mL

add 652 ul of FI-V to 428 ul ALHYDROGEL + ABSORBED FOR 2 HR @ 4°C

- spun tube @ 2000 rpm for 5 min. & removed 2 100 ul aliquots to check for protein conc. on BCA assay (see BCA #'s results)

- added PBS to absorbed ALH-FIV to 3 mL

FOR GRPS 0A + 0B TOOK FI capsule extract @ 60 EU/mL & conc of 705 ug + added 496 ul of this to 1 mL ALHYDROGEL + 137 ul of thrombin treated Vmaura (5.1 mg/mL) = 49 EU/mL

this is for 10 ug/200 ul dose of FI + 20 ug/200 ul dose V in a final volume of 7 mL

496 ul of FI = 350 ug 137 ul V = 700 ug V

496 ul FI + 137 ul thrombin V + 367 ul PBS + 1 mL ALHYDROGEL

ROCK ON @ 4°C

remove 2 100 ul aliquots for BCA assay

- add PBS to 7 mL final volume

REDACTED (cont)

GRPS 1A + 1B	10 mg FI + 20 mg hV in 7 mL final volume hV = 1.3 EU/mg 496 ul FI (705 ug/ml) + 129 ul hV (5140 ug/mL) + 375 ul PBS + 1 mL ALHYDROGEL, Rock ON @ 40C - Spin 2000 rpm, 5 min. remove 2 100 ul aliquots for BCA Assay of absorption - then added PBS to 7 mL final volume
GRPS 2A + 2B	same as 1A + 1B above but used 750 ul ALHYDR + additional 250 ul PBS
GRPS 3A + 3B	same as 1A + 1B above but used 562.5 ul ALHYDROGEL + 437.5 ul PBS to equal 1 mL then same as 1A + 1B
GRP 4 + 5	added 1 mL ALHYDROGEL to 1 mL PBS + rocked ON. Then added PBS to 7 mL final volume
GRPS 6	added 750 ul ALHYDRO + 250 ul PBS + 1 mL PBS & rocked ON @ 4°C. Then added PBS to 7 mL final volume.
GRP 7	added 562.5 ul ALHYDROGEL + 437.5 ul PBS then 1 mL PBS & rocked ON @ 4°C - added PBS to 7 mL final volume
GRP 8+9	Added 496 ul FI + 129 ul hV + 1.375 mL PBS & rocked ON. Then added PBS to 7 mL final volume.

REDACTED

BCA assay results:

BSA	1:10	.181	.187
	1:20	.079	.096
	1:40	.035	.041
	1:80	.012	.015

FI + hV .14 (2A+2B)

#1	#2
1.000	.500

FI + hV .19 until (1A+1B)

#1	#2
-.007	-.001

FI (+hV) (3A+3B) .10

#1	#2
-.003	-.005

FI + TV .19 until (0A+

#1	#2
-.003	-.002

FI-V ALKYDED .19 (00A+00B)

.004
.003

REDACTED

Sartorius Q15 experiment

- had 5mL of FIV @ ~ 2mg/mL + did Q15 exp. as follows:

1) equilibrated Q15 c̄ 15mL 10mM Tris (pH7.6), 0.5mMEDTA,
2) APPLIED 4.5mL of FIV in 10mM Tris, 0.5mMEDTA pH7.6 to filter (collect "FLOW THRU")
3) WASHED Q15 c̄ 10mL Tris-EDTA equilibration buffer + collected as "Wash"
4) eluted c̄ 12mL Tris, EDTA + 100mM NaCl + collected
5) " " " " " + 200mM NaCl " "
6) " " " " " + 300mM NaCl " "
7) " " " " " + 400mM NaCl " "
8) " " " " " + 500mM NaCl " "

ran 10% Tricine gel of all collected parts (next pg.)

Exhibit DH19

AIBS PEER REVIEW TO USAMRMC MEDICAL BIOLOGICAL DEFENSE RESEARCH PROGRAM ON PLAGUE

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APPROVED: _____ DATE: March 12, 1996
Tom Schwan, PhD
KATHLEEN McDONOUGH, P.H.D.

AIBS PEER REVIEW TO USAMRDC
MEDICAL BIOLOGICAL DEFENSE RESEARCH PROGRAM
ON PLAGUE

TIME: February 15, 1996, 8.00am to 5.00 pm

LOCATION: US Army Medical Research Institute of Infectious Diseases, Conference Room, Fort Detrick, Frederick, MD

EXECUTIVE SUMMARY

Overall, the program has made very significant and impressive advances in only a few years towards the development of a new vaccine, and Dr. Friedlander and his entire team of investigators can be proud of their accomplishments to date. They clearly have a very viable, sound program with a good team of investigators that is focused with high potential to succeed. It is hoped that the administration will continue to support this effort and provide the group with the resources and time necessary to complete their task. The investigators clearly considered the recommendations of the previous reviewers and incorporated several of the suggestions into their program.

The team has invested significant effort in examining numerous virulence determinants of *Yersinia pestis* for their ability to stimulate protection through immunization. The F1 capsular antigen and the V antigen have been shown by investigators in other laboratories to be good candidates for inclusion in a new multivalent subunit vaccine. The team at USAMRMC has confirmed the protective value of these two antigens. However, realizing that F1 and V antigens might not be sufficient for full protection against all virulent strains of *Y. pestis*, the group has worked through an impressive list of additional candidates. The only other antigen that offered significant protection was YopD, although protection was only observed when mice were challenged with the $F1^-$ strain. Passive immunizations with anti-F1 and anti-YopM antisera deserve further attention. Combined antibiotic treatment and immunization might increase the survival of animals challenged by aerosol.

The team appears to make use of mice and nonhuman primates as excellent animal models for both their parenteral and aerosol challenge experiments. The current vaccine study protocols for test challenges are very good.

The development of *in vitro* correlates of immunity should be a high priority of the program. It is currently the weakest portion of the future plans. As discussed with the investigators, the assumption that protection is solely antibody-mediated has potential difficulties. Before continuing studies to map active B cell epitopes, the investigators need to determine the role of T cells in immunity to plague.

INTRODUCTION

AIBS was requested by US Army Medical Research and Development Command (USAMRDC) to convene a review Panel to provide an assessment of the scientific merit of the Medical Biological Defense Research Program (MBDRP) on Plague. It was requested that the three scientific reviewers have a collective knowledge of the following subject areas: *Yersinia pestis*, Vaccine Production, Molecular Genetics and FDA requirements for a vaccine. Such a panel was convened and provided with documentation by USAMRDC to read prior to the conference. This consisted of abstracts prepared by the individual investigators who form the MBDRP on Plague (see Appendix 1.)

CHARGE TO PANEL

Three scientific reviewers were asked to evaluate the MBDRP on Plague. They independently reviewed material provided by USAMRDC and attended a conference on the subject matter. They were asked to judge the scientific merits of the Program.

The reviewers, individually, provided comments to AIBS, who in turn compiled this written report summarizing these comments and the discussions at the conference. The Chairman of the Review Panel read and approved the report prior to its submission to USAMRDC.

PRESENTATION SUMMARIES

The conference comprised presentations by each of the following investigators. Abstracts were provided for by each and are attached as Appendix 1.

COL ARTHUR FRIEDLANDER
Overview of plague program

COL RUSSELL BYRNE
Antibiotic treatment of experimental pneumonic plague

DR. PATRICIA WORSHAM, DR. M. LOUISE PITT, LTC KELLY DAVIS
F1 is not a required virulence factor for the mouse or non-human primate

MAJ GERALD P. ANDREWS, LTC GEORGE J. ANDERSON, JR.
Protective efficacy of active immunization with purified F1 from *Yersinia pestis* and an *Escherichia coli* recombinant strain against lethal parenteral and respiratory plague challenge

DR. PATRICIA WORSHAM
Studies on the role of the pigmentation locus in the pathogenesis of *Y. pestis*

DR. SUSAN L. WELKOS, LTC KELLY J. DAVIS
Analysis of the role of pPst encoded genes in pathogenesis of infection by *Y. pestis*

DR. ALAN SAMPLE
Plasminogen activator protease degrades proinflammatory cytokines

MAJ GERALD P. ANDREWS, DR. SUSAN STRALEY, DR. ALAN SAMPLE, MAJ GERALD P. ANDREWS
Cloning, Expression, Purification, and Protective Efficacy of Yops and pH 6 antigen

LTC GEORGE J. ANDERSON, JR., DR. DAVID HEATH
Cloning, expression, and protective efficacy of V antigen

LTC GEORGE J. ANDERSON, JR.
Cloning, expression, and protective efficacy of F1-V fusion protein

DR. JEFFREY PULLEN
Determination of important B and T-lymphocyte epitopes in the F1 and V antigen proteins of *Yersinia pestis*

COL ARTHUR FRIEDLANDER
Overview of future plans

SUMMARY EVALUATIONS OF THE RESEARCH AREAS

The review panel read the abstracts provided by the investigators prior to the meeting on February 15, 1996, and listened to presentations by each of the investigators at the meeting. The following comments include recommendations to individual investigators, and are intended to be constructive. Certain points apply to more than one project, or even to the program as a whole, and hence may appear repetitive. Also, the reviewers recognize that some of their recommendations may be affected by programmatic decisions that are beyond the control of the immediate Program staff and thus may not prove to be possible.

COL ARTHUR FRIEDLANDER
Overview of plague program

The USAMRMC Plague Research Program's primary objective is to develop a vaccine that will protect military personnel if exposed to an aerosol attack of *Yersinia pestis*, the causative agent of plague. Given that the currently available vaccine (USP) protects primarily through anti-F1 antibody, that this vaccine offers very poor protection from primary pneumonic plague, and that $F1^-$ strains are highly virulent, there clearly is a need for a new, more protective vaccine. Once developed, the general population living in areas endemic for plague would also benefit from such a vaccine.

Most of the projects presented as separate studies and presentations clearly meet the program's primary objective. Part of the rationale for the approach taken is that an aerosol attack of *Yersinia pestis* might include strains that do not produce the F1 capsular antigen. Given that the current vaccine (USP) stimulates primarily antibodies

examined for *Y. pestis* in the LD50 studies and survivors were examined for clearance of the organisms to determine the full level of protection provided by vaccination.

In the first study, the V antigen was examined for its ability to generate a protective immune response in mice challenged by parenteral subcutaneous or aerosol challenge with either the $F1^+$ or $F1^-$ isogenic strains of *Y. pestis*. Recombinant V antigen was cloned and expressed in two fusion/expression systems and used with an adjuvant approved for human use (Alhydrogel). Both preparations of rV antigen were administered twice and provided very good protection in mice challenged by both routes and both strains. This is an excellent study and identifies (as another laboratory has demonstrated independently) the V antigen as an excellent candidate immunogen to include in a vaccine to protect from aerosol infections with either $F1^+$ or $F1^-$ strains. These studies are critical to the program's objective and provides some quite exciting results.

The second study extends the work on the V antigen of *Y. pestis* by examining protection following a single dose of 10 μg (the previous study used two immunizations prior to challenge). Mice were subsequently challenged by aerosol exposure to either low and high doses of the $F1^+$ or $F1^-$ strain. Protection ranged from 70% to 78% survival in these mice, demonstrating that a single immunization could afford significant protection from an aerosol route of infection. However, the schedule including a primary immunization followed by a single boost afforded 20% to 30% greater protection (previous report). While it is of interest what level of protection results from a single dose, future work with nonhuman primates will likely confirm what we know about many other bacterial vaccines, i.e., better protection results with boosts following the primary immunization.

Two areas need to be addressed in future work on the V antigen. The studies presented used the V antigen tagged with histidine from the pET vector. If this antigen is to be used in humans, a method for the efficient removal of the his-tag is needed. Identifying the active sites on the V antigen responsible for protective immunity as well as potential negative biological activities, such as immune suppression, may be required for this antigen to be safe. The group might also consider examining how long protective immunity lasts following vaccination with the V antigen. Some of these issues were addressed by Dr. Friedlander in his closing remarks.

LTC GEORGE J. ANDERSON, JR.
Cloning, expression, and protective efficacy of F1-V fusion protein (abstract 17)

Prior studies have confirmed the potential for both F1 and V antigen to protect mice from *Y. pestis* by both parenteral and aerosol routes. In this study a construct was made containing the F1 and V antigen genes for expression of a fusion protein. When the F1-V fusion protein was used for immunization, mice were protected when challenged by needle or aerosol with either the F1 positive or F1 negative strain of *Y. pestis*. Poorer protection resulted when only a portion of the V antigen was expressed as a fusion protein with F1. This work is quite clever and interesting, and advances the program's effort towards the development of a multivalent vaccine. The attempt to make fusions of these two antigens also demonstrates an advance towards reducing

the steps required for making and purifying antigens for the vaccine. The investigators are also testing longer term antibody responses and how long protection lasts (a concern raised from the previous studies with the V antigen alone). Antibody responses to the F1 and V antigen components of the fusion protein were also examined. Both F1 and the V antigen have been shown by other workers to be protective and now the group at USAMRMC has shown that rF1 and rV are the best candidates identified to date for a new plague vaccine.

Again, this fusion protein has a histidine tag, which will need to be removed prior to its use in humans.

DR. JEFFREY PULLEN
Determination of important B and T-lymphocyte epitopes in the F1 and V antigen proteins of *Yersinia pestis* (abstract 18)

This study attempts to identify important B and T cell epitopes within both the F1 and V antigens, however only B cells were addressed in the presentation. Identifying the functional epitopes in these proteins is important both to an understanding of the protective mechanisms stimulated by these two immunogens, and for assessing the potential of using synthetic peptides rather than entire recombinant proteins in a vaccine. This study is an important part of fulfilling the long-term objective of developing a useful vaccine. However, the usefulness of the current appproach should be carefully reconsidered.

The use of short peptides to generate antibodies without conjugation to carrier molecules has, in general, not been very successful. Although it is sometimes possible to generate antibodies against short peptides, it is unlikely that the response will be protective without some T cell involvement. The investigators' initial experiments showed that peptides generated from the region of the protein known to be antigenic failed to generate a protective response despite generating significant antibody production. These results should have alerted them to the problems inherent in this approach. Instead, the investigators expanded their studies in response to these findings by making and testing additional peptides covering the whole of V antigen and F1 protein. This was a lot of work, using a lot of mice, that generated very little useful information. A simpler and more direct approach to begin mapping the reactive epitopes in these immunogens is to screen the overlapping peptides *in vitro* using antisera from animals or humans that have either had infections with *Y. pestis* or been immunized with native F1 and/or V antigen. Another concern is that in the future goals, it was stated that the response to the peptides, rather than to the native antigen will be tested to better determine the response. However, since the goal is to get protective antibodies, it seems that the reponse to native antigen, which is what the animals will see in an actual infection, is what should be measured.

It is also important for the investigators to determine the nature of a protective immune response to *Y. pestis* infection before restricting their focus and undertaking such labor-intensive studies to define only B cell epitopes. Antibody reactivity does not assure protection, and with some pathogens high antibody titers have even been correlated with disease progression. In addition, non-F1 antigens may evoke a

COMBINED RECOMMENDATIONS AND CONCLUSIONS

The USAMRMC's program to develop a new subunit vaccine for pneumonic plague has been very productive and has made significant advances towards this objective. The leader and research team are highly skilled, competent investigators and, with continued support, it is anticipated that a new vaccine for human trials is only a few years away. The investigators have used very effective immunization and challenge protocols to test immunogens in both mice and nonhuman primates for protection against plague following either parenteral or aerosol exposures to *Yersinia pestis*. Having the facilities to safely execute aerosol transmission studies is a critical component of this program. The team has confirmed and extended the data supporting the potential for both recombinant F1 and V antigens to afford significant protection. The work using the F1-V antigen fusion protein is exciting and represents a significant advance made by this team.

The team has examined numerous other antigens for identifying additional protective immunogens, especially for challenge with strains of *Y. pestis* lacking the F1 antigen. For such isolates, the V antigen and possibly YopD are the only useful candidates identified to date. The addition of one more antigen would likely solve the problem of non-responders, as well as strengthen the response in all individuals. The choice of antigens being tested for potential vaccine components appears somewhat random. These studies could be focused better by determining what proteins induce an immune response, thereby demonstrating which determinants are most likely being seen by the immune system. Although it is not possible to predict in advance which antigens are protective, the search could have been directed more towards antigens known to induce an antibody response in infected human patients and laboratory infected animals. Additional focus on the basis of immunity to plague challenge is also recommended. The investigators are also aware of the immunosuppressive effects of V antigen, and plan to examine the mechanisms involved. These types of studies should allow the team to "fine tune" the V antigen to increase its efficacy and safety as a vaccine component.

The development of *in vitro* correlates of immunity should be a high priority of the program and is currently the weakest area of the future plans. As discussed with the investigators, the assumption that protection is solely mediated by antibody has potential difficulties. Before continuing studies to determine important B cell epitopes, the role of T cells needs to be addressed in collaboration with immunologists. There are standard methods, such as adoptive transfer, to determine if T cells protect against challenge with *Y. pestis*. There are also *in vitro* techniques to determine if T cells taken from an immunized animal proliferate in response to specific antigens. The studies using synthetic peptides have potential, but this work needs to be done with conjugated peptides. Alternatively, peptides could be attached to larger inert particles that could be taken up by B cells or macrophages that then present the antigen on class II MHC molecules on their surface. Epitope mapping of the F1 and V antigen peptides using immune sera from natural infections would have been an appropriate first step.

APPENDICES

APPENDIX 1: **AGENDA**

APPENDIX 2: **ABSTRACTS**

REVIEW OF PLAGUE RESEARCH PROGRAM

USAMRIID

15 FEBRUARY 1996

0815-0830 Welcome and introduction
COL David Franz, DVM, Ph.D.

0830-0900 Overview of Plague Program
COL Arthur M. Friedlander, M.D.

Treatment

0900-0930 Antibiotic treatment of experimental pneumonic plague
COL Russell Byrne, M.D.

Role of F1 Capsule in Pathogenesis and Immunity

0930-1000 Protective efficacy of active immunization with purified F1 from *Yersinia pestis* and an *Escherichia coli* recombinant strain against lethal parenteral and respiratory plague challenge
MAJ Gerard P. Andrews, Ph.D.

1000-1015 Coffee Break

1015-1100 F1 capsule is not a required virulence factor for the mouse or non-human primate
Patricia L. Worsham, Ph.D.
M. Louise Pitt, Ph.D.
LTC Kelly J. Davis, DVM

Role of Non-F1 Proteins in Pathogenesis and Immunity

1100-1130 Studies on the role of the pigmentation locus in the pathogenesis of *Y. pestis*
Patricia L. Worsham, Ph.D.

1130-1300 Lunch

1300-1320 Analysis of the role of pPst encoded genes in pathogenesis of infection by *Y. pestis*
Susan L. Welkos, Ph.D.

1320-1335 Plasminogen activator protease degrades proinflammatory cytokines
Allen Sample, Ph.D.

1335-1405 Cloning, expression, and protective efficacy of Yops and pH 6 antigen
MAJ Gerard Andrews, Ph.D.

1405-1420 Cloning, expression, and protective efficacy of V antigen
LTC George J. Anderson, Jr., Ph.D.

1420-1435 Cloning, expression, and protective efficacy of F1-V fusion protein
LTC George J. Anderson, Jr., Ph.D.

1435-1450 Determination of important B and T-lymphocyte epitopes in the F1 and V antigen proteins of *Y. pestis*
Jeffrey Pullen, Ph.D.

1450-1515 Overview of future plans
COL Arthur M. Friedlander, M.D.

Recombinant F1-V (rF1-V) Fusion Protein Protects against Lethal Wildtype Yersinia pestis in a Mouse Model

DAVID G. HEATH, GEORGE W. ANDERSON, JR., CHRISTOPHER BOLT, SUSAN L. WELKOS, PATRICIA L. WORSHAM, AND ARTHUR M. FRIEDLANDER
Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD.

The virulence of F1- strains and their occurence in nature imply that F1 immunogen will not be sufficient for an optimal new plague vaccine. A fusion protein has the theoretical possibility of simplifying and reducing the cost of production of multiple antigens in addition to stabilizing the protein. For these reasons, we developed a fusion protein consisting of both the F1 and V antigens (1). The first fusion protein made consisted of F1 fused with residues 168-175 of the V antigen, a segment which previous studies suggested to contain a protective epitope. This fusion protein was used with the adjuvant alhydrogel (aluminum hydroxide) to immunize female Swiss Webster (Hsd:ND4) mice subcutaneously (s.c.) on days 0 and 30 followed by a s.c. or aerosol challenge with either the F1- C12 strain (LD50 = 9.1 CFU, s.c.; LD50 = 1.1 x 105 CFU, aerosol route) or the wild-type F1+ CO92 (LD50 = 1.9 CFU, s.c. route; 2.1 x 104 CFU, aerosol route) strain of Y. pestis. Endotoxin had been removed from the preparations prior to immunization, so that this would not be a confounding factor.

When 18.5 µg of the F1-V168-275 fusion protein was used to immunize mice, there was 90% survival (9/10) when challenged s.c. with 63 LD50 of the F1+ CO92 strain. The positive control was a group of mice immunized with 10 µg of rF1 which is equivalent to the F1 content of the F1-V168-275 protein. The rF1 control resulted in 100% (10/10) protection. The F1-ELISA IgG titers were the same (1:81920). All mice in alhydrogel control group died (0/9; MTD ± SD, 5.2 ± 1.0). When the F1-V168-275 immunized mice were challenged with 104 LD50 by the aerosol route, protection was 80% (8/10; MTD ± SD, 20.3 ± 7.1) compared to 0% for the control group (0/10; MTD ± SD, 3.1 ± 0.3; 80-104 LD50). The group immunized with rF1 resulted in 70% protection (7/10; MTD ± SD, 9.0 ± 1.0) when challenged with 80 LD50. The addition of part of the V protein onto the F1 protein did not appear to effect its antigenicity.

The F1- strain, C12, was used to test the ability of the partial V portion of the F1-V168-275 protein to protect mice against a lethal challenge. Here 27 µg of the F1-V168-275 fusion protein was used, which is equivalent to 10 µg of the V protein

known to be protective. A s.c. challenge dose of 55 LD50 resulted in 30% survival (3/10, MTD ± SD, 9.4 ± 7.0). All of the controls died (0/10, MTD ± SD, 10.8 ± 4.8). While there was some protection, there was no increase in the MTD. There was a good V-ELISA antibody response to the F1-V168-275 (1:163840). In case this response was not sufficient, another group was immunized with 27 µg, but with complete Freund's adjuvant (CFA). In this case, protection was only 20% (2/10, MTD ± SD, 9.1 ± 3.2), while 10 µg of rV in CFA resulted in 100% protection. The V-ELISA titer when CFA was used was 1:1310720 for F1-V168-275 and rV. A 10-fold increase in the V-antibody titer did not have any effect on protection and the V-ELISA titer was not indicative of protection. When a group of F1-V168-275 mice were challenged with 95 LD50, C12, by the aerosol route, no mice survived (0/10, MTD ± SD, 3.5 ± 0.5) All of the alhydrogel control group died (0/10, MTD ± SD, 3.4 ± 0.5). In other experiments, rV itself gave 80-90% protection against an aerosol challenge.

These results demonstrated the feasibility of making a F1-V fusion protein. The efficacy of F1 was not altered by making a fusion protein. However, while the V168-275 protein portion of the fusion protein was antigenic, it was not immunogenic. This caused us to address the question as to whether the entire V protein could be fused to F1 and whether it would be immunogenic.

Using a fusion protein which combines the whole F1 and the whole V protein (rF1-V) to immunize mice on days 0 and 30 increased the protection afforded by the V portion of the fusion protein. When 13.6 ug of rF1-V was used to immunize mice, there was 100% (10/10) protection against a s.c. challenge of 57 LD50 and 90% (9/10) protection against 1.1 x 106 LD50 C12 strain. Ten micrograms (10 µg) of rV also gave 90% (9/10) protection against 1.1 x 106 LD50, C12 strain. All of the alhydrogel control group died (0/10, MTD ± SD, 6.0 ± 0.0). The rF1-V protein also offered protection against an aerosol challenge. The same immunization schedule resulted in 100% (10/10) when mice were challenge with 546-636 LD50, C12 strain on day 73 postimmunization. When mice immunized with the rF1-V fusion protein were challenged with 762 LD50 of the F1+, CO92 strain by the aerosol route, 100% (10/10) of the mice survived. The F1-V fusion protein was able to protect mice from a significant aerosol challenge from either a F1+ or F1- lethal strain of Y. pestis. This protection is better than the protection afforded by the current Plague Vaccine USP. When mice which were immunized on day 0 and 30 with 0.2 ml of the current vaccine and challenge by the aerosol route on day 73 postimmunization with 546-636 LD50, C12 strain, all of the mice died (0/8, MTD ± SD, 3.3 ± 0.5). The V-ELISA titer to the Plague

Vaccine USP was <1:640.

The recombinant rF1-V fusion protein was produced in E. coli and contained a polyhistidine tag which aids in the purification of the fusion protein. While this protein has been shown to be highly efficacious in the mouse model, it remains to be seen whether this level of protection will be seen the in the non-human primate model. Further, the regulatory issue of whether a histidine tagged protein will be acceptable to the Food and Drug Administration needs to be resolved.

1. Heath, D.G., G.W. Anderson, Jr., J. M. Mauro, S.L. Welkos, and A.M. Friedlander. Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. manuscript submitted.

2. Brubaker, R.R., A.K. Sample, D.Z. Yu, R.J. Zahorchak, P. C. Hu, and J.M. Fowler. 1987. Proteolysis of V antigen from Yersinia pestis. Microbial. Pathogenesis. 2:49-62.